

Multiplex Protein Quantitation using iTRAQ® Reagents – 8plex

Overview

The advent of mass spectrometry based tagging methods, in particular iTRAQ® Reagents, have permitted relative expression measurements of large sets of proteins with a high degree of automation. The isobaric nature of the tags allows the protein samples to be pooled post labeling without increasing the complexity of the MS analysis. Identical peptides labeled with the different iTRAQ® reagents exhibit the same parent ion in MS. Upon MS/MS fragmentation of the parent ion, unique signature ions are generated which distinguish the individual samples and hence the relative abundance among the samples can be determined (Figure 1).

As this iTRAQ® reagent technology has become established and gained acceptance in relative protein analysis, there is a requirement to expand the scope of this technique to enable a higher degree of multiplexing. Described herein is a new set of reagents that doubles the

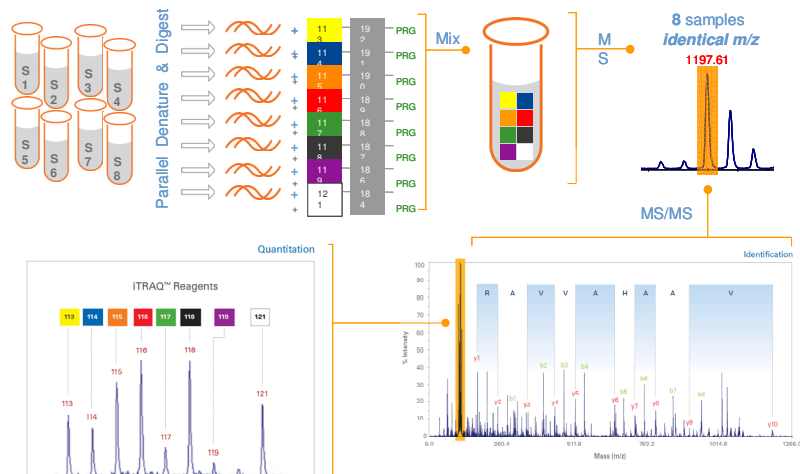


Figure 1. Workflow Once labeled with the iTRAQ Reagents – 8plex, the 8 individual samples are then be pooled for further processing and analysis. During subsequent MS/MS of the peptides, each isobaric tag produces a unique reporter ion that identifies which samples the peptide originated and its relative abundance.

number of states that can be compared from 4 to 8 using the same robust NHS chemistry and easy to use protocols as the iTRAQ® Reagent - 4 plex. This provides greater flexibility in the design of experiments, for example:

- A control and up to 7 experimental samples
- Examination of up to 8 states in a time course study
- Generation of an absolute quantitation calibration curve
- Statistical support by inclusion of duplicates or triplicates within the same sample

The effectiveness of these new reagents to quantitate eight states simultaneously was determined against a set of known peptides and proteins. The reagents were evaluated for label efficiency, fragmentation efficiency, and precision and accuracy of quantitation.

Methods

iTRAQ® Reagents – 8plex Labeling

Protein samples (50 µg) were dissolved in 20 µl of TEAB (triethylammonium bicarbonate (pH 8.5), reduced with TCEP tris-(2-carboxyethyl)phosphine) and alkylated with MMTS

(methyl methanethiosulfonate). Trypsin was added and the samples digested overnight. The eight iTRAQ[®] Reagents – 8plex were prepared by adding 70 µl of ethanol to each vial. The resulting solution was then transferred to each corresponding sample tube and incubated at room temperature for 2 hours, unless otherwise noted. The samples were then mixed and submitted for analysis.

Analysis of the iTRAQ[®] Reagents labeled Samples

Labeled samples were analyzed by either electrospray or MALDI.

Electrospray analysis was performed by diluting the labeled peptides in 0.1% formic acid, 2% acetonitrile and injected (Agilent 1100 System) onto a 75 µ x 15 cm C18 PepMap column. Peptides were eluted from the column using a gradient and analyzed on a QSTAR[®] Elite-Hybrid ESI LC-MS/MS. Data were collected using Analyst QS 2.0 software.

MALDI samples were prepared by diluting the labeled peptides in 0.1% trifluoroacetic acid, 2% acetonitrile and injected (LC Packings) onto a 100 µ x 15 cm C18 PepMap column. Peptides were eluted from the column using a gradient and spotted on a MALDI-

TOF plate. The plate was then analyzed on a 4800 MALDI TOF/TOF[™] Analyzer. All data were processed for protein identification and quantification using ProteinPilot[™] Software.

Labeling Efficiency

The peptides *LNENIR, *ETLDPSAPK* and *LSLGLLQPEK*PVVLK* (* denotes potential labeling site) were prepared for labeling as per the protocol. The iTRAQ[®] Reagents – 8plex – 113 was then transferred to a tube containing 34 µg of peptides and allowed to react. After 30 minutes, the entire contents of a tube of iTRAQ[®] Reagents – 8plex – 115 was added to the tube of peptides and allowed to react for 30 minutes. This was repeated with the iTRAQ[®] Reagents – 8plex – 117, 119 and 121 for a total of 150 minutes (2.5 hr). The sample was then analyzed to determine labeling efficiency.

Fragmentation Comparison

Aliquots of an 8-protein mix were labeled with either the iTRAQ[®] Reagents – 4plex according to the 4plex kit protocol or the iTRAQ[®] Reagents – 8plex according to the 8plex kit protocol. These samples and an unlabeled aliquot of the 8-protein mix were analyzed

by LC-MS/MS. The fragmentation patterns of the peptides were then compared.

Accuracy and Precision of iTRAQ[®] Reagents – 8plex

Two proteins, ovotransferrin and carbonic anhydrase were spiked at varying concentrations into 8 vials of a 6-protein mix (beta-galactosidase, serum albumin, serotransferrin, beta-lactoglobulin, alpha-lactalbumin and lysozyme) that was kept at constant concentration. The two proteins were added so that the total protein per tube remained constant (~50 µg.) Each of the eight vials was labeled with a different iTRAQ[®] Reagent – 8plex. The experiment was performed in triplicate to determine the reproducibility of the protocol.

Signal Amplification

8 vials of 6-protein mix (40 µg) and 8 vials of ovotransferrin (40 µg) were reduced, alkylated and labeled with a corresponding iTRAQ[®] Reagent – 8plex – 113 to 121. Post labeling, two pools were created. Pool A contained equivalent amounts of each of the 6-protein mix samples labeled with its corresponding iTRAQ[®] Reagent – 8plex (32 µg total) and 40 ng of ovotransferrin, labeled with only the iTRAQ[®] Reagent –

8plex –113. Pool B was prepared the same except 40 ng of each of the remaining ovotransferrin samples labeled with iTRAQ® Reagents 114 to 121 was added. This increased the total amount of ovotransferrin in the sample to 320 ng. The sample was then analyzed and the MS/MS fragmentation of the ovotransferrin peptides compared.

Results and Discussion

The 8-plex reagents were designed to have the same reporter ion structure and NHS reactive group as the iTRAQ® Reagents - 4plex (Figure 2). As a result, the same, easy to use robust labeling protocol is used. Additionally, the reporter ions begin at m/z 113 and increase incrementally by 1 Da to m/z 121, skipping m/z 120 as that mass represents the ammonium ion for phenylalanine. The reporter ion region is illustrated in Figure 3 for the Fibrinopeptide B [Glu1] peptide (GluFib) labeled at a 1:1:1:1:1:1:1:1 and 1:2:2:5:5:2:1:5 ratio and analyzed by MALDI. It can be seen the observed ratios match the expected values very closely.

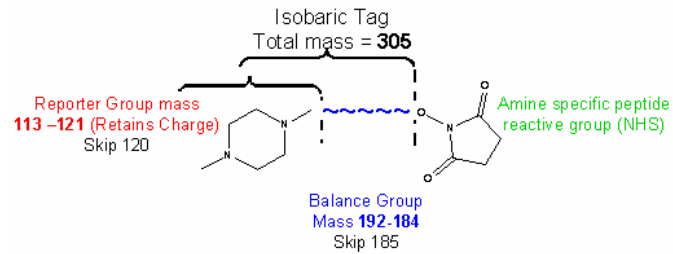


Figure 2. Design of the iTRAQ Reagents - 8plex The 8plex exhibits the same reporter ion structure and NHS chemistry as the 4plex reagent. The balance group had to be changed to allow 8 isobaric tags.

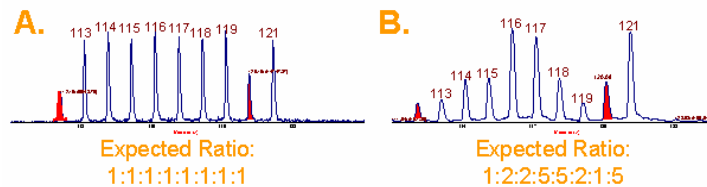


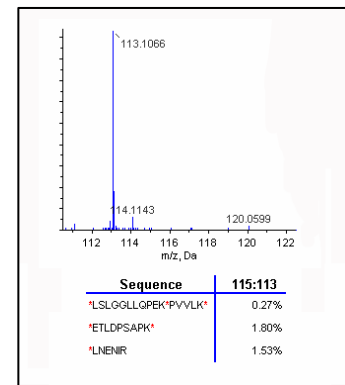
Figure 3. iTRAQ Reagents – 8plex Reporter Ions Expanded signature ion region of the MS/MS fragmentation spectra of GluFib. The signature ions exhibit ratios that mirror the expected ratios.

Labeling Efficiency

The labeling efficiency of the iTRAQ® Reagents – 8plex was determined using a time course study on a 3-peptide mix. Three peptides were selected that contained single, double and triple labeling sites (*LNENIR, *ETLDPSAPK* and *LSLGLLQPEK*PVVLK*.) The 3-peptide mix was first

labeled with the iTRAQ® Reagent – 8plex - 113 for 30 minutes upon which, the iTRAQ® Reagent – 8plex - 115 was added and reacted for an additional 30 minutes. This was repeated at 30 minutes intervals with 3 additional reagents for a total of 150 minutes. Absence of the 115-peak (Figure 4) suggests that the

Figure 4. Labeling Efficiency The reporter ion region (top) from the MS/MS of labeled peptide (*LSLGLLQPEK*PVVLK*) shows the strong presence of the 113 reporter ion and the absence of the 115, 117, 119 and 121 reporter ions suggesting complete labeling after treatment with the 113 reagent. An examination of the relative ratios of the reporter ion for all three peptides (bottom) demonstrates that the reaction was 98% complete after 30 minutes.



reagent labeled both the N-terminal amine and the lysine side chain ϵ -amine to completion within the first 30 minutes. Further analysis of the 115:113 ratio, for all three peptides, indicates that the initial labeling was greater than 98%.

Fragmentation Efficiency

Incorporation of an iTRAQ® Reagent onto a peptide changes the fragmentation profile. To demonstrate the difference, aliquots of the 8-protein mix labeled with iTRAQ® Reagents – 8plex, iTRAQ® Reagents – 4plex and unlabeled were analyzed by electrospray LC-MS/MS. Figure 5

visualizes the difference in fragmentation of the serotransferrin peptide *APNHAVVTR. The most noteworthy difference is that the peptides, once labeled with the iTRAQ® Reagents, exhibit a spectrum rich in both b and y ions. This is in contrast to the unlabeled peptide that is dominated by only the y series. The presence of the reagents can also improve signal intensity as is demonstrated in this example. These two factors combine and lead to higher scoring peptides and greater confidence in the protein identification.

Accuracy and Precision of Relative Quantitation

For any quantitative method to be of value, accuracy and precision of the overall technique must be high. To further validate the protocol, carbonic anhydrase and ovotransferrin were spiked at differing concentrations into 8 vials of a 6-protein mix. The samples were then digested, labeled in triplicate and analyzed by ESI and MALDI. Upon MS/MS fragmentation, the iTRAQ® Reagents labeled peptides generate reporter ions that represent the relative quantitation of that peptide across the samples. An

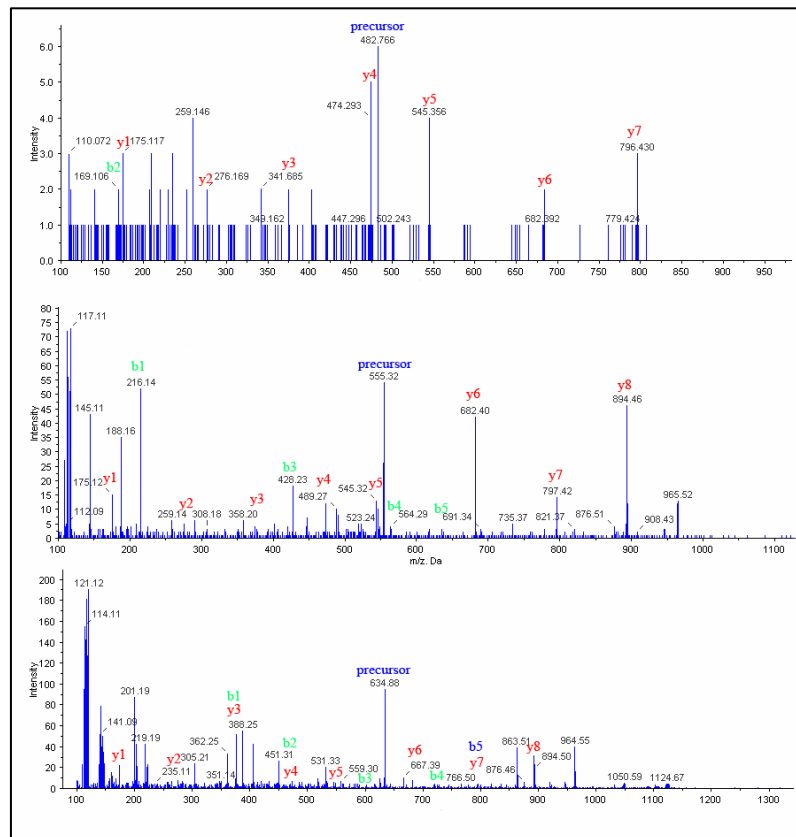


Figure 5. A comparison of fragmentation patterns obtained from serotransferrin peptide APNHAVVTR (top) unlabeled, (middle) iTRAQ Reagent – 4 plex labeled and (bottom) iTRAQ Reagent – 8plex labeled peptides. The iTRAQ Reagent labeled peptides exhibit an improvement in the b ion series.

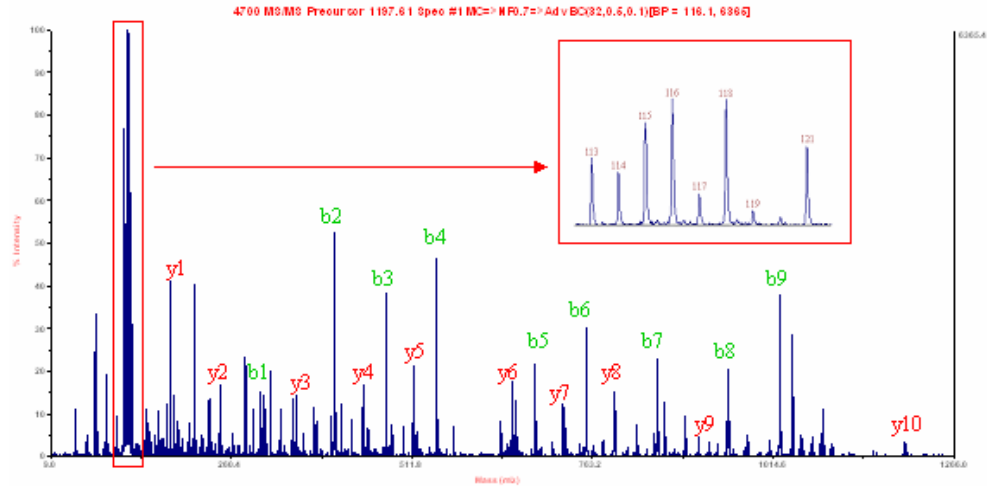


Figure 6. 4800 MSMS of *VAAHAVVAR. This ovotransferrin peptide exhibits ratios (0.7:1.7:1.9:0.6:1.9:0.02:1.3) close to the expected reporter ion ratios of 0.5:1.5:1.8:0.2:1.9:0.1:1.

example of a MALDI-MSMS spectrum from an ovotransferrin peptide (*VAAHAVVAR) is shown in Figure 6. The reporter ions exhibit area ratios (0.7:1.7:1.9:0.6:1.9:0.02:1.3) very close to the expected values of 0.5:1.5:1.8:0.2:1.9:0.1:1.

The resulting ProteinPilot software analysis of this eight protein mixture sample is shown in Table 1. The observed ratios from all eight proteins were close to theoretical. The two differentially expressed proteins, carbonic anhydrase and ovotransferrin, exhibit ratios that closely resemble the expected values of 1.5:0.5:0.2:1.8:0.1:1.9:1 and 0.5:1.5:1.8:0.2:1.9:0.1:1 respectively. In the model system, superoxide dismutase was identified having similar ratios as

Table 1. ProteinPilot Software Result Table The 8-protein mix validation samples were analyzed on the QSTAR® Elite system and processed with ProteinPilot Software. The two differentially expressed proteins, carbonic anhydrase and ovotransferrin exhibit ratios close to the expected ratios of 1.5:0.5:0.2:1.8:0.1:1.9:1 and 0.5:1.5:1.8:0.2:1.9:0.1:1 respectively.

% Cov.	Acc. #	Name	Species	114:113	115:113	116:113	117:113	118:113	119:113	121:113
68.5	P02789	Ovotransferrin	CHICK	0.4961	1.4979	1.5670	0.4066	1.9393	0.1750	1.2011
64.5	P02787	Serotransferrin	HUMAN	0.8449	1.0010	1.0273	1.2841	1.0533	1.0206	1.2310
60.5	P02769	Serum albumin	BOVIN	0.8617	1.0397	0.9350	1.2145	0.9718	0.9142	1.1306
61.8	P00921	Carbonic anhydrase 2	BOVIN	1.3302	0.5243	0.2833	2.3752	0.2607	1.8728	1.3649
59.3	P02754	Beta-lactoglobulin	BOVIN	0.9000	1.2725	0.8940	1.4920	1.0771	1.1449	1.4595
81.6	P00698	Lysozyme C	CHICK	0.9225	1.0291	0.9704	1.2361	1.0222	0.8753	1.0470
40.3	P00760	Cationic trypsin	BOVIN	0.9977	1.0829	0.7757	1.1304	0.9927	0.9114	1.0985
57.6	P00442	Superoxide dismutase	BOVIN	1.5018	0.6325	0.3416	2.3913	0.3387	1.8697	1.4037
19.6	P00722	Beta-galactosidase	ECOLI	1.2979	1.8832	2.2971	3.2505	5.0080	4.8333	5.6502
15.5	P01013	Ovalbumin-related protein X	CHICK	2.2764	2.2045			4.8325		3.0153
27.5	Q9TSN6	Alpha-lactalbumin	BUBBU	0.7302	0.9887	0.7699	1.1568	0.7633	0.7815	1.1577
21.6	P56410	Ovotransferrin	ANAPL		1.5942	1.4158		3.0832		1.7163
80.6	P67976	Beta-lactoglobulin	SHEEP							

carbonic anhydrase. This suggests that the superoxide dismutase is a contaminant in the carbonic anhydrase source.

To obtain a more precise value of quantitation accuracy for this method, std deviation and coefficient of variance were calculated for

all the observed peptides from the same protein

The results for serotransferrin (top 15 scoring peptides) are shown in Table 2. The coefficient of variance was calculated for each of the iTRAQ® Reagents ratios which were expected to be 1:1:1:1:1:1:1. The average coefficient of variance for all eight reagents was 14%. This is consistent with our previous

experience with the iTRAQ[®] 4plex reagent of <20% std deviation.

To determine run to run reproducibility, the three samples were analyzed in triplicate by electrospray. Upon identification and quantitation with ProteinPilot, the coefficient of variance for the relative ratios was calculated within each sample and across all samples (Table 3.). The average coefficient of variance (CV) for the same sample analyzed three times was determined to be around 6.5%. This increased to 14% when calculated across all 9 analyses.

Signal Amplification

One of the key features of the iTRAQ[®] reagents is the isobaric nature of the tag. This creates an amplification effect of low abundant proteins by allowing identical peptides, split between experimental samples, to combine and produce a stronger parent ion. This may often improve the quality of the MS/MS spectra and lead to higher coverage of the identified protein.

In this experiment, a 6-protein mix was labeled with all 8 of the iTRAQ[®] Reagents – 8plex. From this sample, two pools were created. In the first pool, ovotransferrin labeled with only the iTRAQ[®] Reagent –

Table 2. Accuracy of the MS/MS Reporter Ions An analysis of the top 15 peptides for serotransferrin demonstrates the accuracy of the iTRAQ Reagent – 8plex quantitation.

Serotransferrin Expected Ratio 1:1:1:1:1:1:1							
Sequence	114:113	115:113	116:113	117:113	118:113	119:113	121:113
ADRDQYELLCLDNTR	0.912	1.023	0.921	0.991	0.933	0.995	1.162
IECVSAETTEDCIAK	0.963	1.061	0.951	1.005	0.998	0.822	0.991
EGYYGYTGAFFR	0.956	0.920	0.947	1.096	0.972	0.973	1.084
DCHLAQVPSHTVVAR	1.260	0.939	0.915	0.914	0.949	0.909	0.984
SDNGEDTPEAGYFAVAVVK	1.068	1.186	0.790	1.116	0.802	1.042	0.926
SVIPSDGSPVACVK	0.976	0.903	0.890	1.033	1.092	1.082	1.108
DOYELLCLDNTR	0.935	1.014	1.046	0.822	1.050	1.437	0.834
DCHLAQVPSHTVVAR	1.094	0.893	0.967	0.856	0.975	1.255	0.984
DYELLCLDGTFR	1.032	0.897	0.978	1.217	0.731	1.094	1.041
MYLGYEYVTAIR	0.865	0.876	1.133	0.952	1.016	1.176	1.015
DSGFQMNQLR	0.988	0.862	1.007	1.127	0.933	1.117	1.084
MYLGYEYVTAIR	1.037	0.981	0.822	1.116	1.058	0.909	1.096
HOTVPONTGGK	1.169	1.109	0.710	1.153	0.819	1.148	0.974
SVIPSDGSPVACVK	0.930	0.958	1.048	1.151	0.811	1.146	0.988
MDAKMYLGYEYVTAIR	0.822	1.165	0.499	0.999	1.657	1.135	0.718
Average	1.00	0.99	0.91	1.04	0.99	1.08	1.00
Standard Deviation	0.114	0.105	0.156	0.116	0.213	0.153	0.112
Coefficient of Variance	11%	11%	17%	11%	22%	14%	11%

Table 3. Reproducibility The coefficient of variance was calculated for each protein ratio in two fashions. First (top) the 3 injections of a single replicate and second (bottom) all 9 injections of the three replicates.

Protein	Intra-Sample Reproducibility - 3 Runs						
	114:113	115:113	116:113	117:113	118:113	119:113	121:113
Beta-galactosidase	10%	8%	10%	13%	21%	7%	20%
Ovotransferrin	8%	2%	2%	12%	1%	23%	1%
Serotransferrin	2%	7%	6%	4%	2%	7%	2%
Serum albumin	4%	7%	2%	2%	4%	4%	2%
Carbonic anhydrase 2	3%	1%	4%	3%	2%	0%	3%
Beta-lactoglobulin	4%	6%	9%	4%	8%	1%	6%
Lysozyme C	5%	4%	8%	4%	4%	9%	7%
Alpha-lactalbumin	5%	6%	13%	12%	9%	13%	18%

Protein	Inter-Sample Reproducibility - 9 Runs						
	114:113	115:113	116:113	117:113	118:113	119:113	121:113
Beta-galactosidase	34%	12%	12%	14%	29%	21%	26%
Ovotransferrin precursor	12%	8%	7%	13%	21%	20%	10%
Serotransferrin	18%	8%	9%	11%	23%	10%	12%
Serum albumin	13%	6%	8%	13%	21%	5%	9%
Carbonic anhydrase 2	14%	9%	13%	9%	22%	6%	7%
Beta-lactoglobulin	13%	10%	14%	14%	24%	13%	14%
Lysozyme C	9%	12%	9%	15%	19%	7%	8%
Alpha-lactalbumin	25%	8%	15%	11%	25%	16%	14%

8plex - 113 was added. In the second pool, ovotransferrin labeled with all 8 of the reagents was spiked into the pool. This, in total, is an 8x increase in the overall concentration of ovotransferrin however the concentration of ovotransferrin labeled with the 113 - reagent remained constant.

Upon analysis of the two pools by LC-MS/MS (Figure 7), a clear amplification of the MS/MS fragment ions was observed. This almost 10x increase in signal intensity was also reflected in the increase from 29% to 63% in ovotransferrin protein coverage.

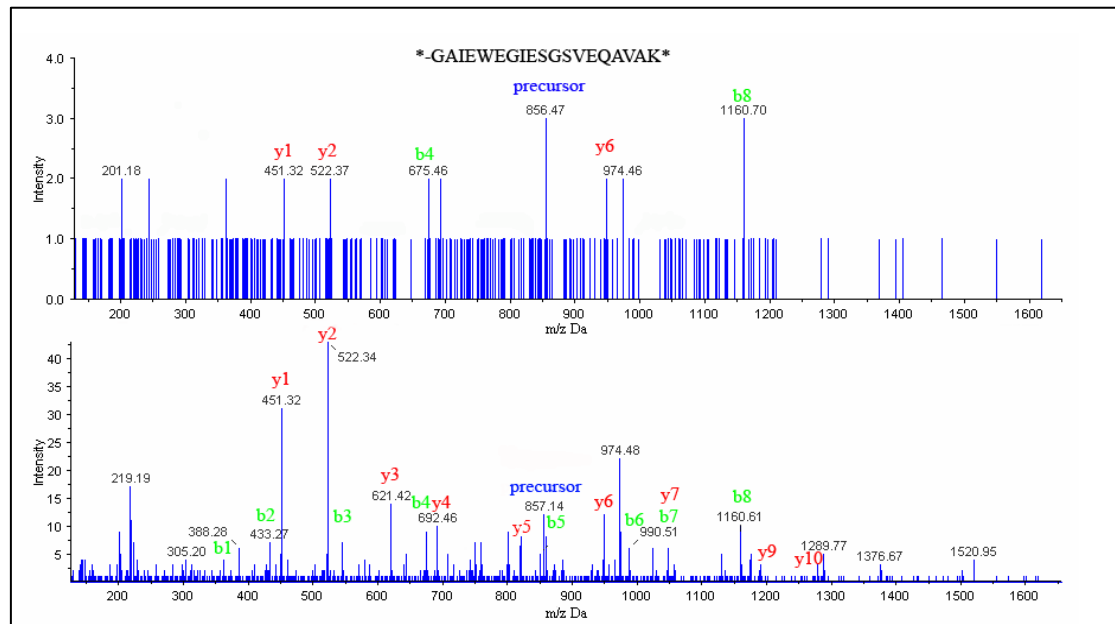


Figure 7. In this signal amplification demonstration, the MSMS fragmentation is shown for an ovotransferrin peptide. The top spectrum represents a pool of peptides in which only ovotransferrin labeled with the 113- reagent has been added. The bottom spectrum was acquired from the same pool with the addition of ovotransferrin labeled with all 8 reagents.

Conclusions

The iTRAQ® Reagent – 8plex are a powerful approach to global protein quantitation. The unique isobaric nature of the iTRAQ® Reagents – 8plex allows for up to 8 samples to be processed simultaneously with a labeling efficiency >98%.

This configuration provides greater flexibility in experimental design whilst vastly improving throughput. Other benefits include the enhanced detection of down regulated proteins due to signal amplification and an improvement in the b-series ions fragmentation of peptides.

Analysis of the quantitation results generated by ProteinPilot Software showed %CV to be less than 20%. These data prove that the iTRAQ® Reagents – 8plex are an effective and reproducible method of quantifying differentially expressed proteins.