iTRAQ™ Reagents

Amine—specific Labeling Reagents for Multiplexed Relative and Absolute Protein Quantitation

Background

Proteomics research includes the characterization of protein mixtures in order to understand complex biological systems and determine relationships between proteins, their function, and protein-protein interactions. Often, the goal is to monitor changes in protein mixture composition under different physiologically relevant conditions, a type of study referred to as differential profiling. While monitoring these qualitative changes is valuable, there is a need to develop quantitative tools that can provide more insight into these experiments. Recent approaches to some of these challenges in protein quantitation involve the combination of chemistry with 2D gel electrophoresis (DIGE) or liquid chromatography (ICAT® reagents). As these techniques become established and gain acceptance in protein expression analysis there is a requirement to expand the scope of these technologies and address some of the remaining issues in quantitative proteomics such as: 1) improve overall protein and proteome coverage while retaining important post translational modification (PTM) information, 2) simultaneously compare multiple samples, e.g. normal versus diseased versus drug treated samples or time course studies, in the same experiment, 3) quantify, in absolute terms, specific proteins of interest, such as biomarkers, or to screen drug targets, such as kinase inhibitors, and 4) increase statistical relevance needed for quantitative experiments by expanding multiplexing, up to four, to include duplicates or triplicates in the design.

These requirements for broad proteome coverage, multiplexing, absolute quantitation, and more statistically significant information has led to the development of the isobaric tags for related and absolute quantitation.

iTRAQ Reagents

Applied Biosystems iTRAQ Reagents are a multiplexed set of four isobaric reagents which are amine specific and yield labeled peptides which are identical in mass and hence also identical in single MS mode, but which produce strong, diagnostic, low-mass MS/MS signature ions allowing for quantitation of up to four different samples simultaneously. In addition to multiplexing, information such as post-translational modifications is not lost using this new chemistry. Since all peptides are tagged, proteome coverage is expanded and analysis of multiple peptides per protein improves the confidence in those identified. Protein identification is simplified by improved fragmentation patterns, with no signal splitting in either the MS or MS/MS modes and the complexity of MS and MS/MS data is not increased by mixing multiple proteome samples together. Additionally, the ability to simultaneously analyze up to four different samples enables the comparison of numerous sample states and provides the flexibility to include

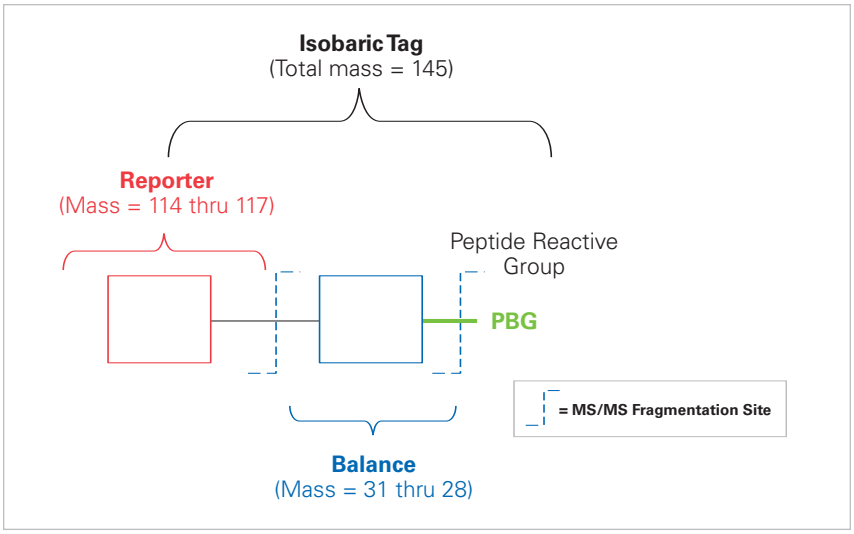

Figure 1. iTRAQ™ reagent structure

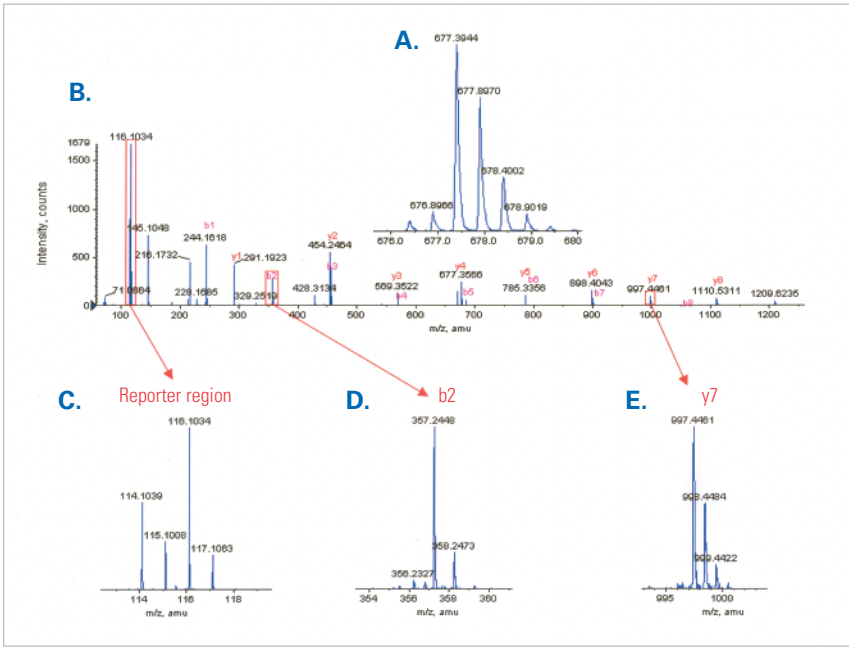

Figure 2. MS and MS/MS spectra from a multiplex sample labeled with 4 iTRAQ™ reagents showing **A.** doubly charged parent ion **B.** MS/MS spectrum corresponding to VLVDTDYK **C.** 4 diagnostic reporter ions and **D.** and **E.** peptide fragment ions

Key Features and Benefits

- Simple workflow labels peptides allowing rapid progression to LC/MS/MS analysis and easy data interpretation with software for relative and absolute quantitation
- Expand proteome coverage by labeling all peptides, including those with post-translational modifications (PTMs) to extract more detailed information from samples
- Analysis of up to four different biological samples simultaneously in a single experiment
- Increase confidence in identification and quantitation by tagging multiple peptides per protein to gain more statistically significant information
- Perform absolute quantitation across numerous sample states, for the synchronous uniform comparison of normal, diseased and/or drug treated states
- Enhance low-level analysis as a result of the signal amplification from the additive fragmentation of labeled isobaric peptides

The iTRAQ Reagent Structure

iTRAQ Reagents are non-polymeric, isobaric tagging reagents consisting of a reporter group, a balance group, and a peptide reactive group as shown in Figure 1.

The peptide reactive group covalently links an iTRAQ Reagent isobaric tag with each lysine side chain and N-terminus group of a peptide, labeling all peptides in a given sample digest. Combining multiple iTRAQ Reagent-labeled digests into one sample mixture, the MS of the mixture resembles the MS of an individual sample (assuming the same peptides are present) as shown in Figure 2A. The balance group ensures

duplicates or triplicates into experimental design, giving the statistical relevance needed for quantitative experiments. The technology has been incorporated into a simple workflow involving parallel protein extraction and routine enzymatic digestion common to any LC/MS experimental procedure. Subsequent labeling of peptides occurs in a manner that allows rapid transfer to LC/MS/MS analysis and easy data interpretation with iTRAQ™ quantitation and identification software (e.g. Pro QUANT software for QSTAR® and Q TRAP® Systems and GPS Explorer™ 3.0 for the 4700 system) without burdensome sample handling requirements. The use of these labels therefore permits simultaneous measurement of relative and/or absolute protein abundance of multiple samples in a single LC/MS/MS run.

that an iTRAQ™ reagent-labeled peptide displays the same mass, whether labeled with iTRAQ reagent 114, 115, 116, or 117.

During MS/MS, the isobaric tag cleaves at the sites indicated in Figure 1 along with the usual peptide fragmentation. As a result of fragmentation, there is neutral loss of the balance group, and the iTRAQ Reagent reporter groups are generated, displaying diagnostic ions in the low-mass region between m/z of 114-117. Because this region is free of other common ions, quantifying the peak areas of these resultant ions represents the relative amount of a given peptide in the respective sample. These diagnostic ions are the key components that make these reagents superior for use in quantitative experiments. Also, an additive series of peptide y- and b- ions are generated and used for protein identification. The combination of these results is illustrated in Figure 2B–E. Mass spectrometric comparison from the MS/MS spectra generated from samples labeled with any of the four different iTRAQ Reagents provide quantitative information on the differences among, up to four different, samples.

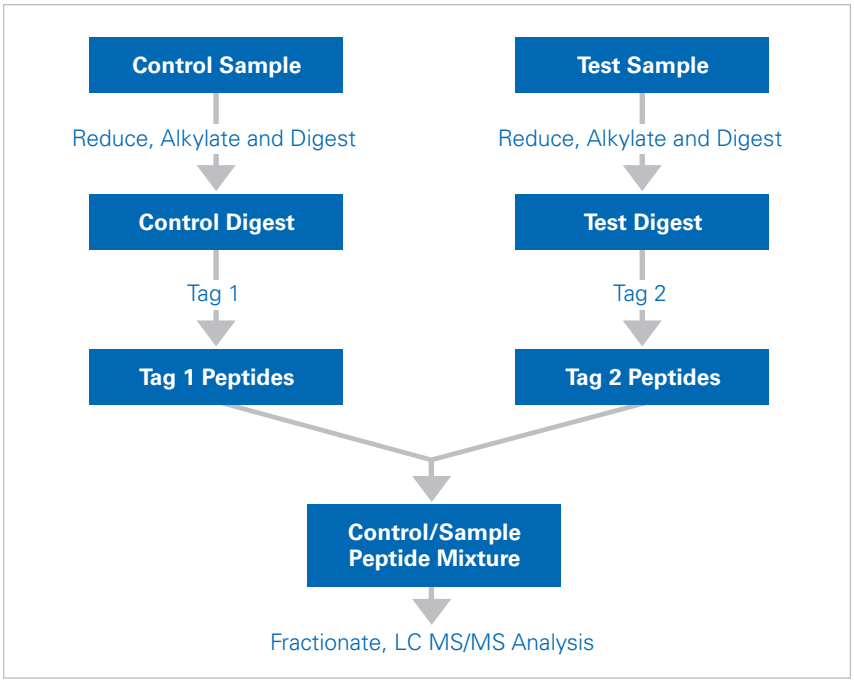

Figure 3. Duplex example of the iTRAQ™ reagent protocol.

The iTRAQ Reagent Protocol—Duplex Example

Although up to four different samples can be analyzed in any given experimental procedure, for simplicity, Figure 3 shows an experiment using only two. Protein isolates are reduced, alkylated and digested with trypsin in an amine free buffer system, in parallel. After simple adjustment of the digest to 70% organic to control any hydrolysis, the resulting peptides are then labeled with the iTRAQ Reagents. Upon completion of labeling the samples are then combined. Depending on sample complexity, samples are either directly analyzed via LC/MS/MS after a one-step elution from a cation exchange column to remove reagent by-products or, in the case of complex samples, cation exchange chromatographic fractionation to reduce overall peptide complexity.

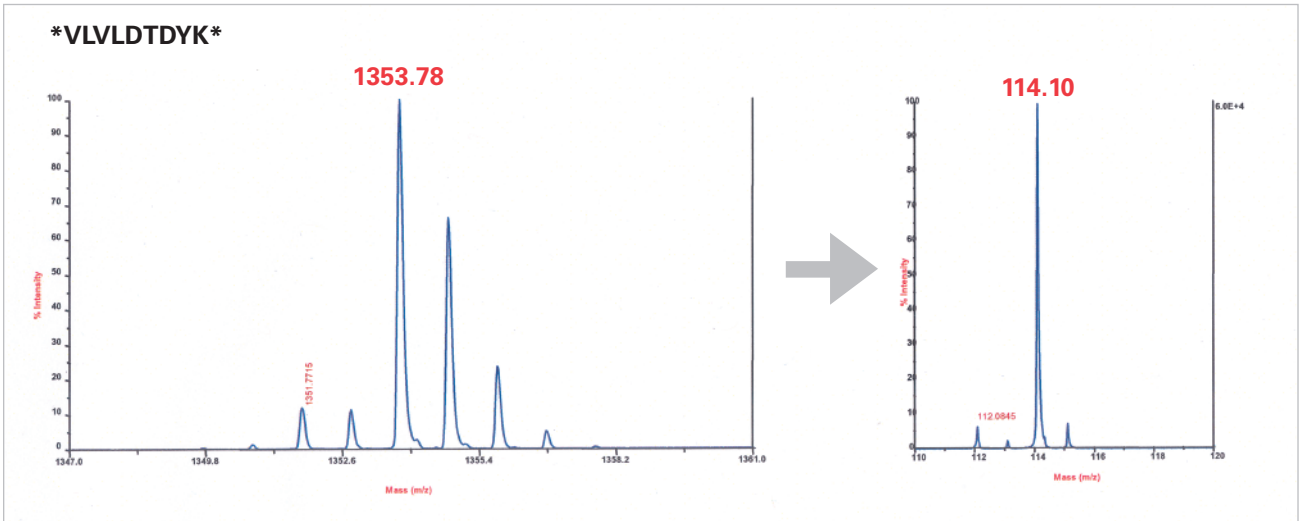

Figure 4. Efficiency of labeling shows absence of 117 after complete initial labeling with 114.

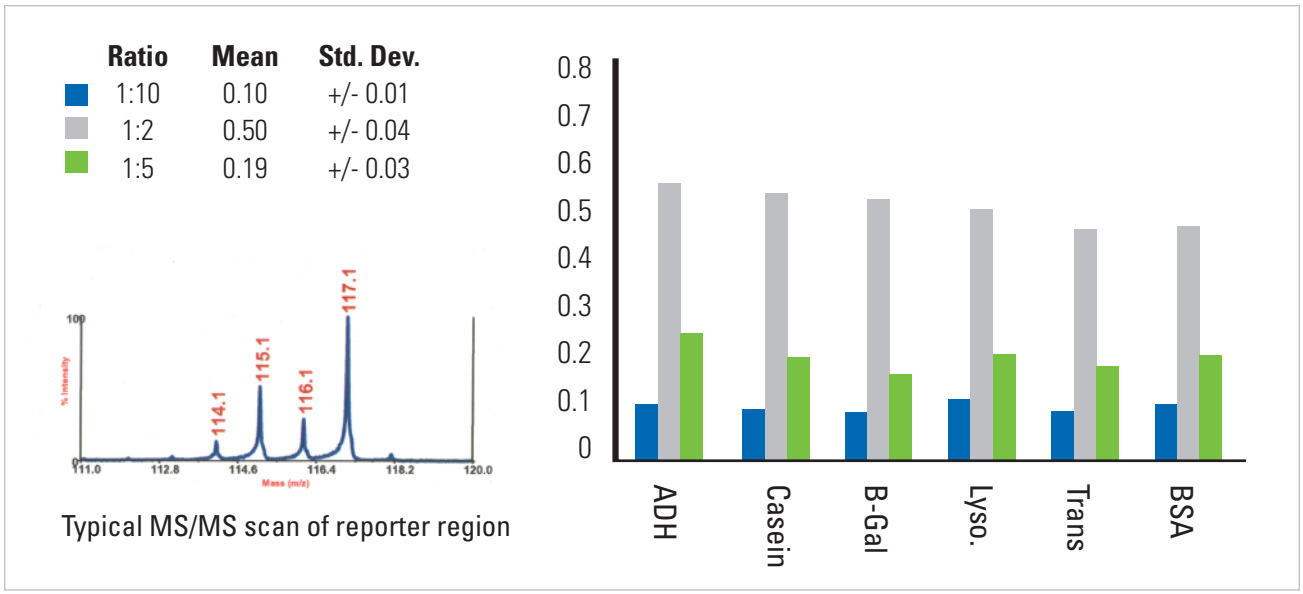

Figure 5. Peptide average grouped by protein.

Protocol Validation

Efficiency of labeling

To determine the efficiency of labeling, a digest of a simple protein mixture was labeled with iTRAQ™ reagent 114. The reaction was allowed to proceed for 60 minutes at room temperature. The resulting labeled peptide pool was subjected to a second labeling, using the 117 reagent. The resulting MS/MS scan of one peptide, VLVLDTDYK from that mixture shown in Figure 4, shows the complete absence of any 117 signal indicating there was complete labeling of both the N-terminal amine and the lysine side chain, ε-amine in a single addition of the reagent and was efficiently labeled with the initial reaction.

Accuracy and Precision in Relative Quantitation

As processes using the iTRAQ™ chemistry involve parallel workflows (up to four different samples), accuracy and precision of the overall technique must be high. A 6-protein mixture was labeled with iTRAQ reagents 114-117 in parallel and mixed in ratios 1:5, 1:2 and 1:10 and quantified by comparing the differences in intensities of the signature ions at 116, 115, 114 and 117, (Figure 5). As illustrated from the data, the relative ratios are accurate compared to the expected values and the standard deviations are < 20% for each of the six different proteins analyzed.

Improved Fragmentation

iTRAQ Reagents not only efficiently label all peptides regardless of class, but also enhance the overall quality of the resulting MS/MS spectra, e.g. enhanced fragmentation. The MS/MS spectrum in Figure 6 shows the difference in spectral quality between samples with and without iTRAQ Reagent labeling.

As readily seen, the spectrum are rich in both y- and b- ions upon tagging while without tagging the spectral signal is dominated by y- ions only and with reduced signal intensity.

Improved Protein Coverage

The improved MS/MS spectra that results from the tagging with the new chemistry, shown in Figure 6, allows for more peptides per protein to be assigned. As shown in Table 1, multiple peptides for every protein in this simple protein mixture were used to assign identification with overall protein coverage improving from a range of 12 to 83%. The increased number of peptides leads not only in an increase in percent protein coverage but also yields an improvement in more statistically relevant quantitation values as well.

Complex Sample Analysis—Relative and Absolute Quantitation

To demonstrate the benefits of using iTRAQ Reagents a more complex sample was analyzed in an attempt to discover which proteins were important in nonsense-mediated mRNA decay. This study involved comparing three strains of yeast: 1) Xrn1 protein knock-out (ΔXrn1) strain, 2) Upf1 protein knock-out (ΔUpf1) strain and 3) wide-type strain. Each sample was reduced, alkylated and trypsin digested independently in parallel and labeled with iTRAQ Reagents 114, 115 and 116, respectively. In addition, a select number of unique peptides to target proteins were labeled with the 117 reagent and added in known amounts in an effort to put an absolute measure to any changes occurring relative to the

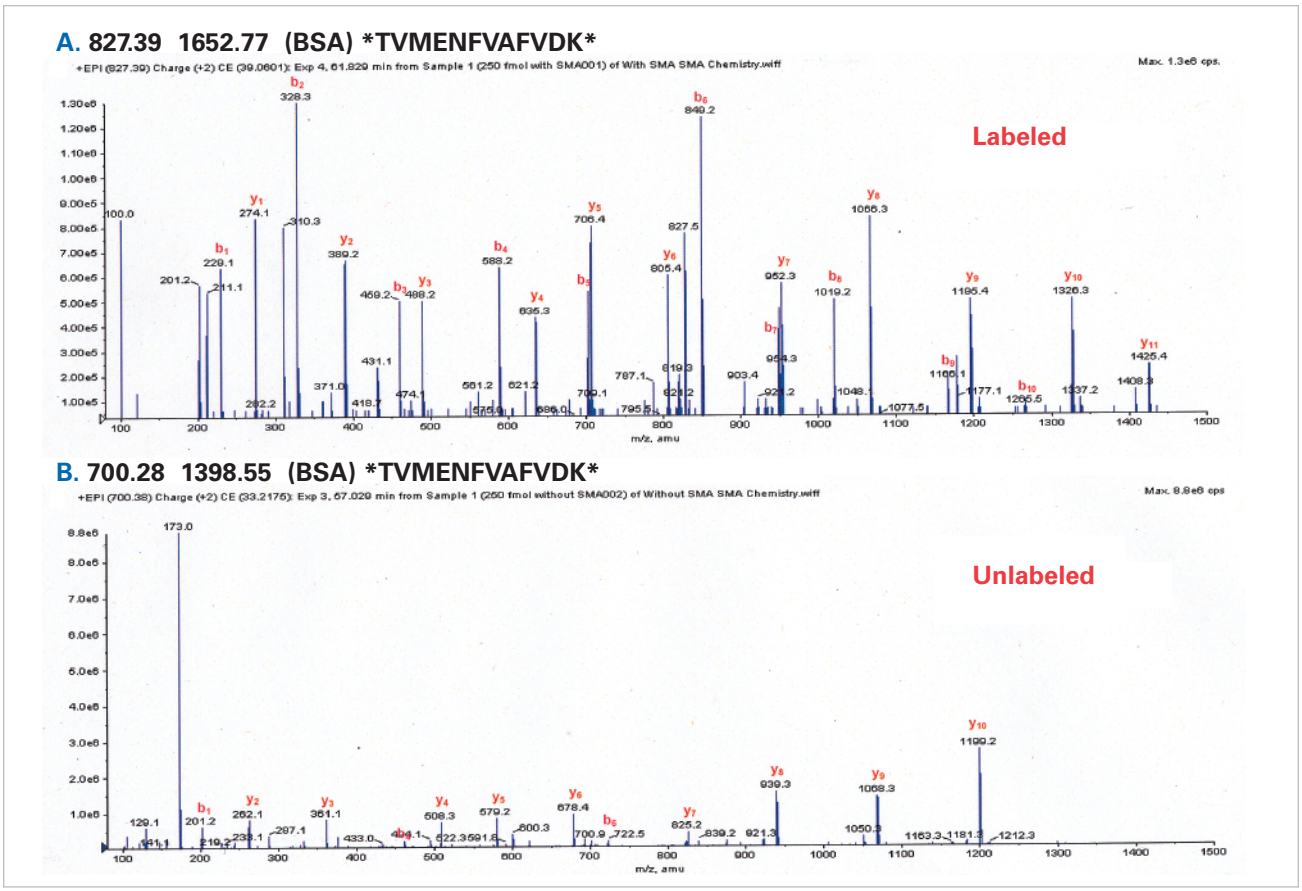

Figure 6. MS/MS Spectra of a BSA peptide TVMENFVAFVDK **A.** with and **B.** without iTRAQ™ reagent labeling.
**iTRAQ reagent labeled residues.*

Example of Proteins Found	% Sequence Coverage		% Increase in Coverage	Quantitation 116 : 114	SD
	Unlabeled	Labeled			
Alpha-Amylase	12.3	20.4	40	1.13	±0.13
Beta-Lactoglobulin	32.0	41.6	23	1.17	±0.01
Bovine Serum Albumin	27.5	43.7	37	1.14	±0.01
Catalase	13.6	36.4	63	1.06	±0.01
Chymotrypsinogen	7.8	22.9	66	1.04	±0.03
Creatine Kinase	33.1	37.8	12	1.02	±0.01
Cytochrome C	10.6	60.6	83	0.94	±0.04
Glucoamylase	8.0	13.8	42	1.07	±0.01
Glutamate Dehydrogenase	18.4	34.1	46	1.00	±0.15
Glyceraldehyde Phosphate Dehydrogenase	22.8	32.1	29	0.96	±0.04
Glycogen Phosphorylase	25.9	34.9	26	1.02	±0.0
Horseradish Peroxidase	10.8	17.6	37	1.02	±0.01
Lactate Dehydrogenase	30.7	44.3	31	1.07	±0.01
Lactoperoxidase	17.8	27.5	35	1.09	±0.02
Lysozyme	33.3	38.1	13	0.99	±0.13
Myglobin	35.9	46.4	23	1.02	±0.17
Ovotransferrin	27.8	32.8	15	1.17	±0.16
Phosphoglucomutase	11.9	31.3	62	1.01	±0.01
Serotransferrin	30.3	50.4	40	1.13	±0.01

Table 1. Protein coverage using a simple protein mixture labeled with iTRAQ™ reagent in a ratio of 1:1.

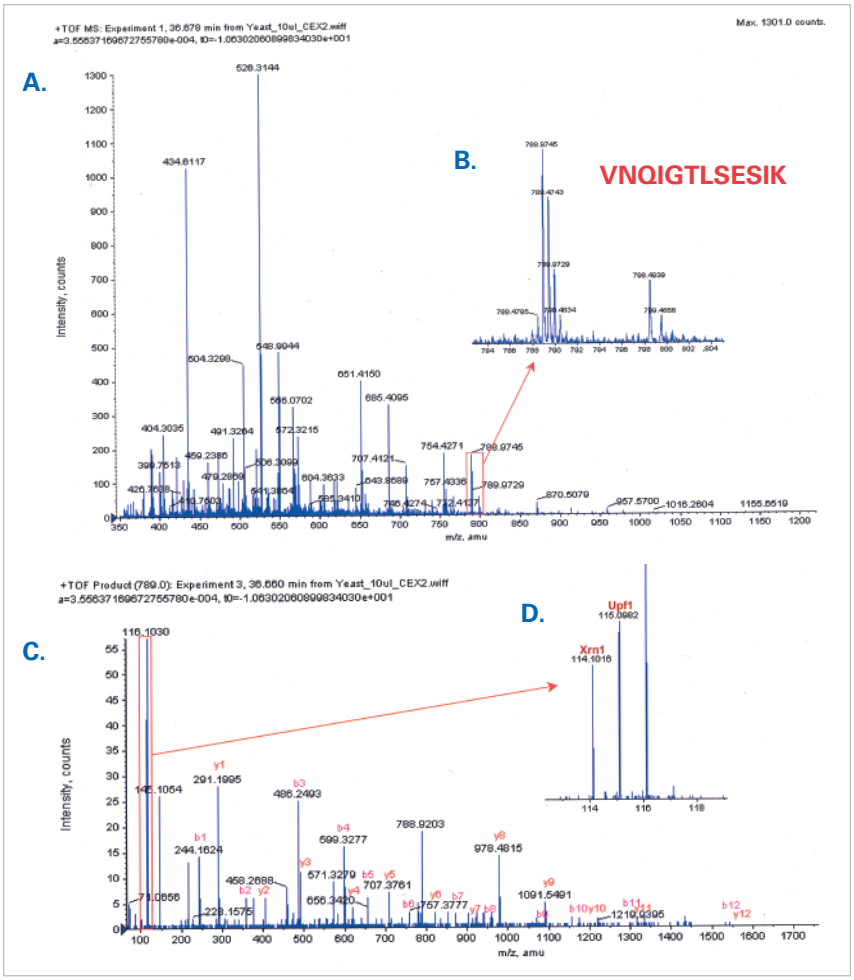

Figure 7. Identification and quantitation of iTRAQ™ reagent labeled peptides using QSTAR® XL MS System showing: **A.** TOF MS Spectrum **B.** Zoomed-in region of TOF MS spectrum of doubly charged parent Ion 788.975 **C.** Corresponding MS/MS Spectrum to peptide VNQIGTLSESIK **D.** Zoomed-in region of MS/MS showing diagnostic reporter ions

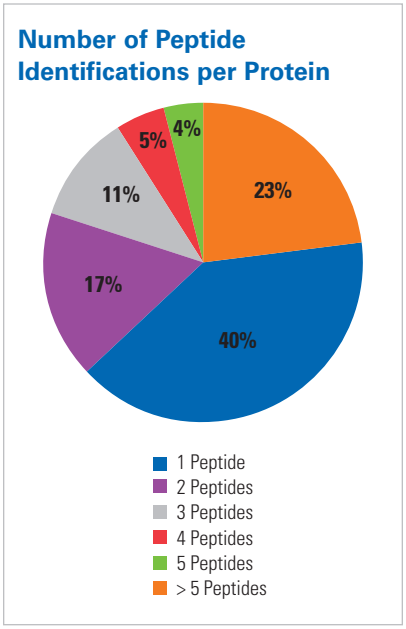

Figure 8: Number of peptide identifications per protein.

wild-type strain. The 4 samples were mixed and then subjected to strong cation exchange (SCX) chromatography. Gradient elution was optimized and 11 separate fractions were collected and analyzed by LC/MS/MS techniques. A representative set of MS and MS/MS spectra are illustrated in Figure 7, analyzed using a QSTAR® XL MS system.

Comprehensive analysis of data collected from both the QSTAR XL System and the 4700 Proteomics Analyzer for the nonsense-mediated mRNA decay study determined that from ~ 4,500 unique high-scoring peptides identified using a strict criteria of data (> 95% confidence interval) yielded 1,217 unique proteins identified with at least one significant peptide, 685 with at least 2 significant peptides (~60%) and < 2–4% of high-scoring peptides had any partial labeling or modifications. The overall average of peptides assigned to any given protein was four. This data is summarized in Figure 8.

In order to determine which proteins were up/down regulated ratios calculated were normalized relative to the wild type. Ratios considered ±1σ unit away from the mean were considered significant as standard deviations were found to be less than 20%. Using this criteria, 39 proteins were determined to be down-regulated in ΔXrn1 mutant and 23 in the ΔUpf1 mutant. Conversely, there were 48 and 62 proteins up-regulated in the ΔXrn1 and ΔUpf1 mutants, respectively.

Quantitation Reproducibility

More statistically significant and confident information can be gleaned by using multiple peptides which contribute, not only to the identification of any given protein, but also confirm the quantitative evidence by averaging multiple peptides together rather than relying on any single value as shown in Figure 9.

Absolute Quantitation

Quantitation of specific proteins of interest can also be performed using the iTRAQ™ Reagents. This involves comparing peptide(s) from a target protein of interest to known (quantified) amounts of labeled standard peptides, which represent unique fragments of that protein, spiked into the sample. In the yeast study described above, a unique peptide to carbamoyl-phosphate synthetase, ILESHDVIVPPEVR, was spiked into the triplex mixture. The reporter region for that peptide is illustrated in Figure 10.

From experiments such as this that involve idiotypic peptides, both relative and absolute quantitation can be obtained in the same MS/MS spectrum. By spiking in additional peptides, statistically significant information can be gleaned by having replicate information in much the same manner as that illustrated in Figure 10.

Software for Data Analysis

The Pro QUANT software package supporting the iTRAQ™ Reagents is available for the QSTAR® XL and Q TRAP® family of MS systems and GPS Explorer™ 3.0 for the 4700 Proteomics Analyzer. With enhanced cleaner MS/MS fragmentation of the labeled peptides, more accurate quantitation and identification is obtained for up to four multiplexed samples. The software is optimized to provide accurate peptide quantitation of the low-mass diagnostic signature ions.

When multiplexing, the reference sample may be user-defined and quantitative results presented with statistical analysis, including data normalization by the median expression ratio. Additionally, it also enables the comparison between sample results and raw data with easy flexible access to all sample results stored in a back-end relational database.

As part of an integrated solution consisting of iTRAQ reagents and the QSTAR XL and Q TRAP family of LC/MS/MS Systems with Pro QUANT Software and the 4700 Proteomics Discovery System with GPS Explorer™ Software, direct access to molecular functions, biological processes, and other biologically relevant information is enabled by linking protein results to the Celera Discovery System™ Online Platform's comprehensive, annotated genomic database.

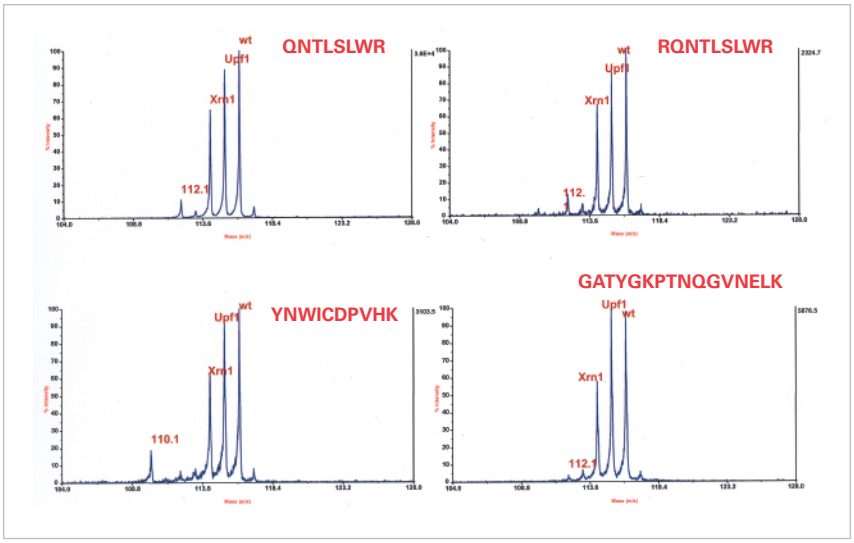

Figure 9. Reproducibility in quantifying the down-regulation of 60S ribosomal protein L15A by 4 peptides.

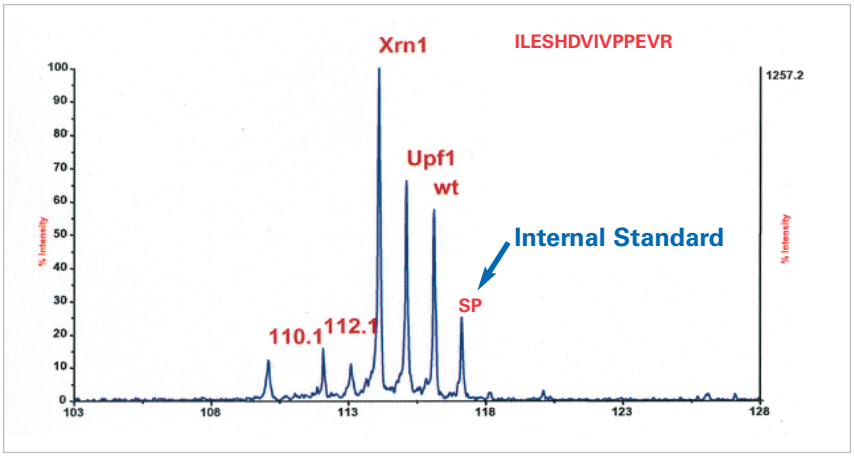

Figure 10. Spiked-in internal standard peptide labeled with iTRAQ™ reagent 117 provides absolute quantitation.

Summary and Conclusions

These reagents have been shown to label all peptides, regardless of class, including PTMs, increasing the overall protein coverage. They work to simultaneously compare up to four different samples enabling a more powerful set of experiments to be undertaken. A very compelling extension of the multiplexing is the ability to run replicates of a given sample and/or control in any single experiment gaining additional statistical significance to any experimental outcome. In addition, the iTRAQ Reagents are versatile in their ability to provide quantitative information from experiments involving affinity pull-downs, time course analysis, membrane protein studies, discovery and validation analysis for biomarker elucidation, and absolute quantitation of target proteins of interest.

These reagents have the flexibility to label diverse sets of samples ranging in complexity from simple mixtures to whole proteomes. Moderately complex samples require a simple, one-step, clean up to remove excess reaction artifacts and immediately onto an LC/MS/MS experiment. Highly complex samples, such as lysates, require fractionation in order to reduce the complexity of the sample to gain more in-depth information.

Not only can the iTRAQ™ Reagents be applied to a wide variety of biological applications, but the kits are designed to provide flexibility in experimental design. From a single multiplex kit one can undertake any combination of duplex, triplex or four-plex experiments.

Whether performing protein expression analysis or absolute quantitation experiments, Applied Biosystems iTRAQ Reagents coupled with our MS systems provide the features, the confidence, and the statistical relevance you need for quantitative biology. In addition to PTM analysis, these reagents are also ideal for laboratories performing discovery/validation analyses for biomarker elucidation, and time course studies.

Ordering Informaion

Description	Quantity	Part Number
iTRAQ™ Reagents Methods Development Kit— Contains iTRAQ Reagents 114 and 117 and sufficient material to develop a robust protocol and verify that sample preparation procedures do not interfere with labeling and digestion.	1 kit	4352160
iTRAQ Reagents Multiplex Kit— Contains sufficient iTRAQ™ Reagents (114, 115, 116, and 117) for 10 duplex, 6 three-plex, or 5 four-plex experiments.	1 kit	4352135
Trypsin 10-Pack— Contains sufficient enzyme for 10 duplex, 6 three-plex, or 5 four-plex experiments.	1 pkg	4352157

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