Protein and Biomarker Quantitation Using iTRAQ™ Reagents

A Novel Set of Multiplexed Amine-Specific Tagging Reagents

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INTRODUCTION

Technologies exist today both gel based (DIGE) and LC-based (ICAT® Reagent Labeling) for relative quantitation of protein expression. While these offer a breakthrough in this area for quantitation, new technologies must address issues such as global peptide labeling (other than cysteine), retaining post-translational modification (PTM) information, mulitplexing (more than 2 samples) and simple workflows which will provide researchers with a much deeper understanding of biological samples.

A new class of isobaric reagents, iTRAQ™ reagents were designed with these concepts in mind. The unique reagent design allows for labeling of samples with 4 reagents of the same mass (isobaric) but upon fragmentation in MS/MS, gives rise to 4 unique reporter ions (114 thru 117) which are used for quantitation of the 4 samples, respectively. Enhanced fragmentation of these labeled peptides in MS/MS also enables more confident protein identification, see Figure 1.

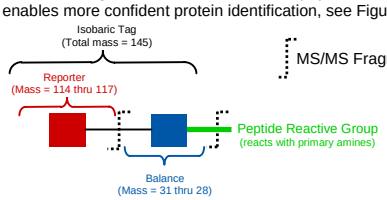
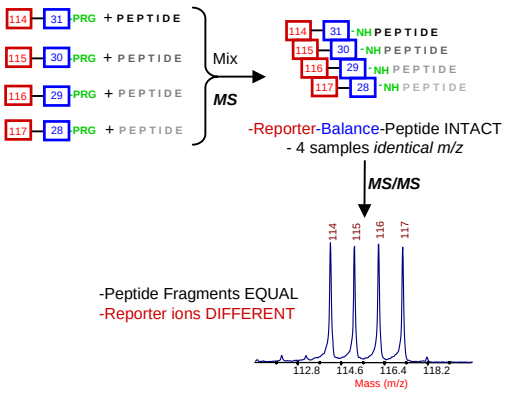


Figure 1. iTRAQ Reagent Structure. The iTRAQ reagent consists of a charged reporter group (unique to each of 4 reagents), a peptide reactive group (labels all N-termini and lysine side-chains) and a neutral balance portion to maintain an overall mass of 145.

All peptides in up to four different biological samples may be labeled simultaneously, enabling relative and absolute quantitation from signature ions produced in MS/MS spectra upon fragmentation, see Figure 2.

Figure 2. The Concept of iTRAQ™ Reagent Chemistry (Example of a 4-plex Experiment). Each sample is labeled with one of the four iTRAQ Reagents and then pooled prior to MS analysis.



These reagents provide flexibility in experimental design. We show 3 examples from experiments involving

1) Time course analysis of drug kinase inhibitor,

2) Determination and quantitation of true binders in and affinity pull-down experiment

3) Discovery, identification and quantification of putative biomarkers in saliva.

Time Course Study to Determine Effect and Magnitude of a Drug Candidate on a Target Protein

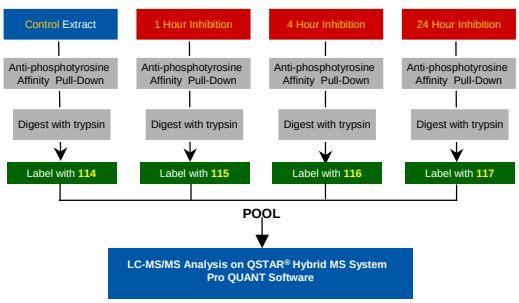
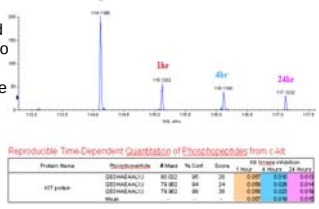


Figure 3. Workflow Showing Time Course Inhibition by a Tyrosine Kinase Inhibitor of Phospho-Tyrosine Complexed Proteins.

The effects of a drug candidate on a target protein (Kit protein) were studied over 4 time points (0, 1, 4 and 24 hours). Figure 3 shows the workflow employing iTRAQ™ reagent labeling and utilizing affinity pull-down strategies.

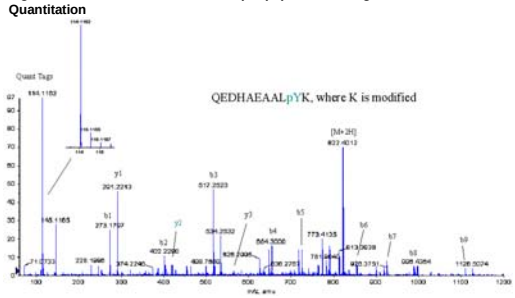
Figure 4. Quantitation of pY Selected Kit Peptide using iTRAQ™ Reagents

Fragment ion spectra of Kit peptides from 4-plex labeled sample following exposure to Kit/KDR inhibitor or control (C) is shown in Figure 4. The reproducibility of this quantitation was also demonstrated.



As all peptides are labeled using these reagents, multiple peptides per protein are used both to identify the protein as well as to quantitate. 21 peptides were identified for the kit protein, each with quantitative data which adds statistical validation to the results (data not shown). The phosphopeptide derives from position 681, within the kinase domain of kit. Figure 5 shows the identification and quantitation of the Kit phosphopeptide over the time course studied.

Figure 5. MS/MS of 821.9m/z kit Phosphopeptide Showing Identification and Quantitation

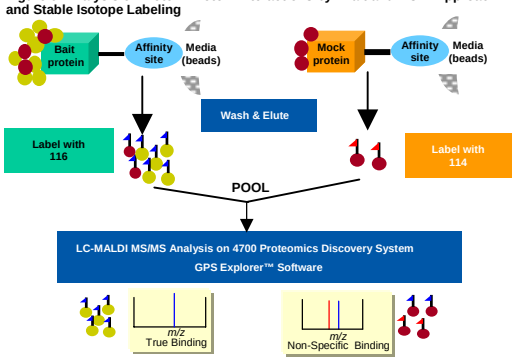


Multiplexing over 4 time points enabled over 200 proteins to be identified and quantified in this study. Retention of PTM information allowed for the study of drug inhibition of phosphoproteins. Several proteins involved in the kit signal transduction pathway were also identified and quantified, such as c-fes/c-fps, SYK and ERK proteins (data not shown). This data supports the elucidation of signaling pathways.

Identification of Protein Interactors in Multi-Protein Complexes

Systematic studies of protein-protein interactions have been reported on using a wide array of bait proteins to pull out interacting partners followed by a tandem affinity purification methods. [1]. However, a significant number of non-specific interactors (false positives) can still remain with the complex. To facilitate the discrimination between true and non-specific interactions of grb2 (Figure 6) and simplify interpretation of pull-down results we have adopted the use of the iTRAQ reagents to reduce the rate of false positives in an effort to achieve more high-throughput, automated workflows.

Figure 6. Analysis of Protein-Protein Interactions by "Bait and Fish" Approach and Stable Isotope Labeling



The iTRAQ™ reagent ratio, calculated at the MS/MS level, allows for a measure of the relative quantitation with true specific interacting proteins having a ratio biased towards the heavy iTRAQ™ reagent. Figure 7 shows an example of a typical true binder, measured at approximately 38.2.
Conversely, non-specific interacting proteins have ratios close to 1:1. An example shown in Figure 8, shows the identification and quantitation of protein carbonyl reductase.

Figure 7. True Interaction Appear as Singlets in MS/MS.

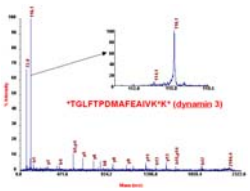
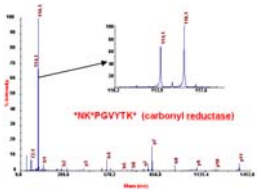


Figure 8. Non-Specific Interactions Appear as Pairs in MS/MS.



Again, several peptides per protein were used both for identification and quantitation therefore increasing confidence in the results. For example, 26 peptides were used to identify and gain an average quantitation ratio for *dynamin 3* (data not shown).
Using this strategy, many known non-specific interactors of the grb2 pull-down process and true interactions to grb2 or grb2-protein complexes (from HeLa cell lysates) were determined (data not shown).

A number of new and unknown protein interactions were found and are under current investigation.

Identification and Quantitation of Salivary Biomarkers

The salivary biome is a good candidate for discovery research of diagnostic markers and therapeutic agents as it is less invasive and easy to collect. Differences in composition and changes in saliva (relative ratios) over a 12 hour period were studied (Circadian Rhythm) using iTRAQ™ reagents to study the natural salivary peptidome (<10kDa), as shown in Figure 9.

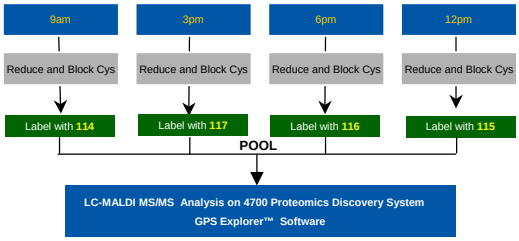


Figure 9. Workflow used to Study Saliva Biomarkers

The relative concentration of some peptides changed dramatically depending on collection time, see Figure 10, where peptide 1 increased in abundance over the 12 hour interval whilst peptide 4 decreased in abundance over the same time interval.

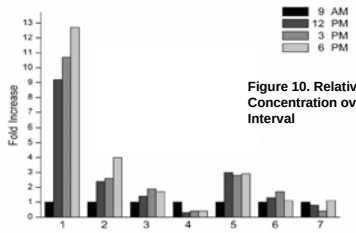


Figure 10. Relative Changes of Peptide Concentration over 12 Hour Time Interval

Once again, multiplexing allowed the study of four time points. iTRAQ reagent labeling enabled much improved MS/MS fragmentation (data not shown) which is important for the study of native peptides which are non-tryptic and often highly charged or large and thus do not fragment well without labeling. Native peptides are often found in serum and plasma also. Salivary native peptides showed considerable dependence on Circadian rhythm and future research in saliva biomarkers need to take into account this phenomenon.

CONCLUSIONS

- iTRAQ™ reagents label all peptides whilst retaining PTM information
  - Important in the study of phosphoproteins and signal transduction pathways
- Multiplexing capability enables the study of :
  - Drug inhibition over 4 time-points
  - Expression of salivary peptides also in a time-course study and
  - Replicates within one LC-MS/MS experiment to add statistical validation
- Labeling enhances MS/MS fragmentation enabling more confident peptide/protein identification
  - Especially important in the identification and quantification of native non-tryptic peptides such as those found in saliva, serum and plasma
- Quantitation and identification are both performed in MS/MS
  - Enables simultaneous ID and quantitation of biomarkers in a single experiment
- Quantitation software is available for iTRAQ reagent technology
  - Pro QUANT software for QSTAR® and Q TRAP® LC/MS platforms
  - GPS Explorer™ for the 4700 Proteomics Discovery System
- Reagents are versatile in providing quantitation information from experiments involving:
  - affinity pull-downs
  - time-course analysis
  - discovery/elucidation of biomarkers and
  - absolute quantitation using labeled internal standards

REFERENCES

1. Gavin, A. C., M. Bosche, et al. (2002). Nature 415(6868): 141-7.