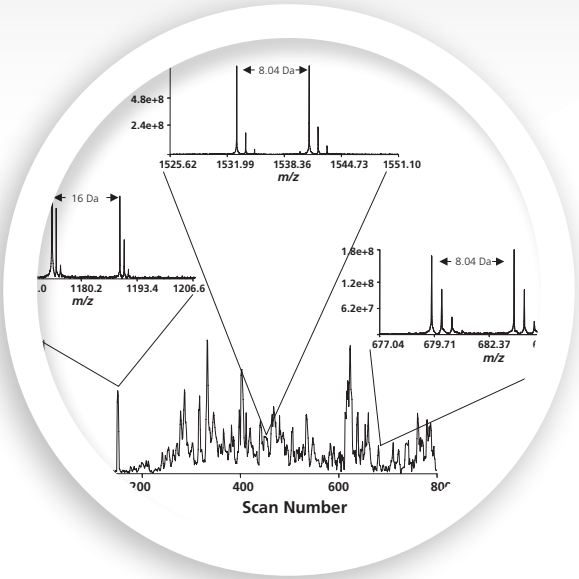
Cambridge Isotope Laboratories, Inc.
www.isotope.com

Eurisotop
A CAMBRIDGE ISOTOPE LABORATORIES COMPANY

APPLICATION NOTE 11

Stable Isotope Labeling in Proteomics

Thomas P. Conrads and Timothy D. Veenstra*
Environmental and Molecular Sciences Laboratory
Pacific Northwest National Laboratory, Richland, WA 99352 USA



Proteomics, the analysis of the proteins expressed by a cell, tissue or organism under a specific set of conditions, has undergone a tremendous period of growth in the past few years. Proteomic studies are typically designed to analyze hundreds or thousands of proteins in a single analysis and aim to provide a global view of changes in protein expression that occur in different cellular growth states or when the cell is treated with a given agent or regimen. While proteomics is formally defined as the complete characterization of the protein complement of a cell, including post-translational modifications, a great deal of intellectual effort has been focused on developing methods to globally measure changes in relative protein abundances between two distinct cell systems (i.e., control vs. treated).

The driving technology supporting proteomic investigations has been mass spectrometry (MS). The ability to rapidly identify proteins and their high sensitivity are just two of the key features of MS that has made it invaluable in proteomics. While changes in protein expression have typically been studied by first separating samples of interest on two distinct two-dimensional polyacrylamide gels (2D-PAGE) followed by comparing the intensity of the Coomassie- or silver-stained spots between gels, this method has many deficiencies related to reproducibility, proteome coverage, and quantitation. Fortunately, there have been several recent developments in the use of stable isotope-labeling strategies that allow combined, yet isotopically distinct, proteome samples (from different sources) to be analyzed.

While mass spectrometry has not been historically used for measuring relative protein abundances, developments in the area of stable-isotope labeling are now making this scenario feasible at both the intact protein and peptide level.[1,2] One of the earliest demonstrations of isotopic-labeling strategies for whole proteomes was the analysis of intact proteins to examine the cadmium (Cd2+) stress response in *Escherichia coli*. In these studies, *E. coli* was grown in both normal (i.e. natural isotopic abundance) and rare isotope (^{13}C,^{15}N)-depleted media.[1] Changes in relative protein abundances were measured by removing equal aliquots of cells from the unstressed (normal medium) and stressed (depleted medium) cultures at different time intervals after Cd2+-addition,

(continued)

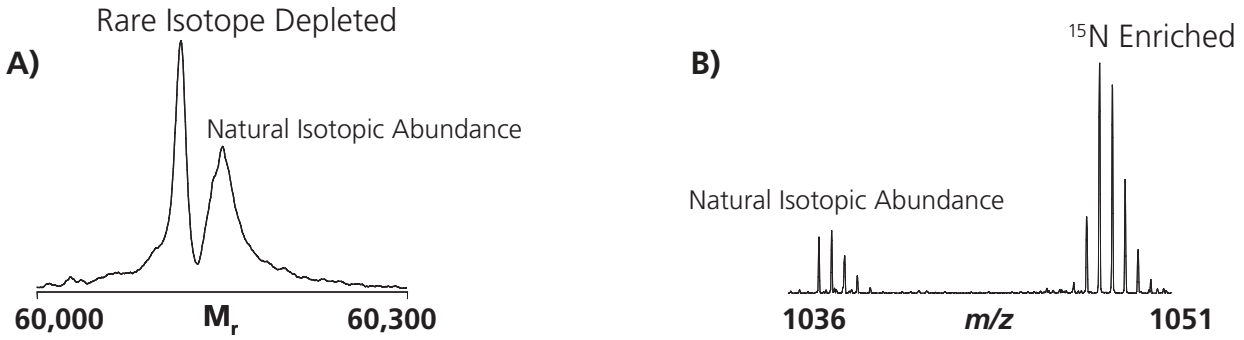


Figure 1. Examples of stable-isotope labeling of an (A) intact protein and (B) peptide observed in the MS analysis of an *E. coli* and *Deinococcus radiodurans* proteome samples, respectively. The two isotopic versions of each were obtained by culturing the cells separately in normal and either isotopically depleted (A) or ^{15}N-enriched (B) media. Combining the two separate cultures provides two isotopic versions for every species present in the samples.

mixing them prior to sample processing and the extracted proteins were analyzed by capillary isoelectric focusing coupled on-line with Fourier transform ion cyclotron resonance (FTICR) MS. In other stable isotope-labeling approaches, cells have been cultured in ^{15}N-enriched medium and combined with cells cultured in normal medium and changes in relative abundance measured by analyzing the peptides produced from a proteolytic digestion of intact proteins.[2] In both of these metabolic-labeling methods, two isotopically distinct versions of each protein (or peptide) are observed, and the relative abundance of the specific protein is quantified by comparing observed peak intensities of each species in the mass spectra, as shown in Figure 1.

While the metabolic-labeling method described above is limited to cells that can be cultured in specifically formulated media, other isotope-labeling methods have been developed that are applicable to proteome samples isolated from any conceivable source. One of the most exciting developments in the use of stable-isotope labeling to quantify changes in the expression of proteins in proteome studies is the isotope-coded affinity tags (ICAT) method.[3] In the application of ICAT labeling, shown in Figure 2, proteins are modified with a cysteine-specific reactive group that covalently modifies reduced Cys residues. The ICAT reagent also contains a biotin tag, allowing the specific isolation of the modified Cys-containing peptides using immobilized avidin. Changes in the relative abundance of peptides from distinct proteome samples is accomplished by the use of isotopically distinct versions of the ICAT reagent; a light isotopic version and a heavy isotopic version in which eight protons in the linker region between the thiol reactive group and the biotin moiety of the ICAT reagent have been substituted with eight deuterons. ICAT labeling results in both stable isotope-labeled Cys-polypeptides, which can aid identification by providing an additional Cys sequence constraint, and provides a significant reduction in complexity of the polypeptide mixture to be analyzed.

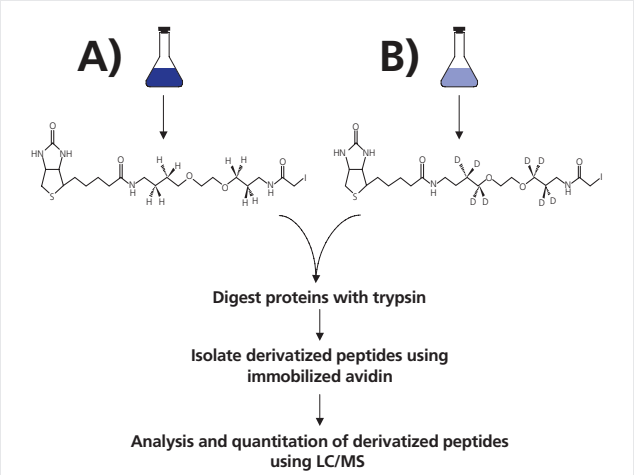


Figure 2. Schematic representation of the isotope-coded affinity tag (ICAT) strategy. Proteins are separately extracted from cells grown under two different conditions (A and B). The proteins for each sample are labeled either with the light (ICAT-D0) or heavy (ICAT-D8) ICAT reagent. After labeling the proteins are pooled and digested with trypsin. The modified peptides are isolated by affinity chromatography and analyzed by capillary LC/MS.

To demonstrate the ICAT strategy, a protein extract from cultured mouse B16 melanoma cells was divided into two equal aliquots. One aliquot was derivatized with the light isotopic version of the ICAT-D0 reagent and the other with the ICAT-D8 reagent. The derivatized proteomes were pooled, digested with trypsin, and the labeled Cys-polypeptides isolated using avidin affinity chromatography. The peptide mixture was analyzed in a single capillary LC/MS experiment by FTICR. In this single analysis, hundreds of pairs of Cys-polypeptides with the expected integral mass difference of 8.0 Da were observed. A few of these peptides are shown in Figure 3. The average ratio of peak areas for the distinct isotopically labeled versions of each peptide was ~1.01.

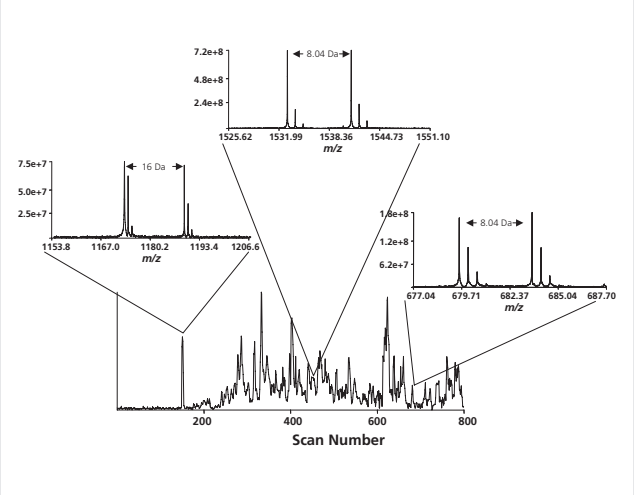


Figure 3. Examples of ICAT-labeled peptides observed in the analysis of mouse B16 melanoma cells. In this analysis a single proteome sample extracted from the cells was split into two equal aliquots that were then labeled with either ICAT-D_0 or ICAT-D_8.

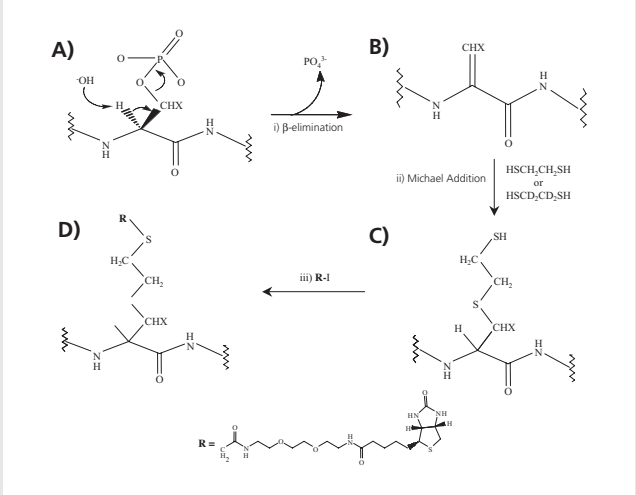


Figure 4. Phosphoprotein isotope-coded affinity tag (PhIAT) labeling method. Proteins containing phosphoseryl (X = H) or phosphothreonyl (X = CH_3) residues are isotopically labeled and biotinylated. After proteolytic digestion, these biotinylated peptides are isolated from non-phosphorylated peptides via avidin affinity chromatography. The ability to quantitate the extent of phosphorylation between two identical peptides extracted from different sources is based on the use of a light ($HSCH_2CH_2SH$, EDT-D_0) and heavy ($HSCD_2CD_2SH$, EDT-D_4) isotopic versions of 1,2-ethanedithiol.

Since identical aliquots of the proteome sample were used in this experiment, average ratio of peak areas for the distinct isotopically labeled versions of each peptide was ~01.01, consistent with the expected results.

Recently, an alternative strategy has been developed that combines ^{15}N-metabolic labeling and post-extraction cysteine affinity tagging to isolate and quantitate Cys-polypeptides analogous to the ICAT strategy.[4] To demonstrate this labeling strategy, proteome samples were again isolated from equal numbers of mouse B16 melanoma cells cultured in normal isotopic abundance and ^{15}N-enriched media were labeled with iodoacetyl-PEO-biotin. Iodoacetyl-PEO-biotin contains all of the elements of the light ICAT reagent: a Cys-specific reactive group with a biotin functionality that can be used to isolate derivatized peptides using immobilized avidin. Pairs of differentially labeled Cys-polypeptides were observed whose mass-to-charge *(m/z)* ratio differed based on the number of nitrogen atoms in the peptide. While the use of the PEO-biotin affinity tag to isolate only Cys-polypeptides significantly reduces the complexity of the mixture, the proteome samples still contain a formidable number of peptides.

While stable isotope-labeling methods have been used to measure differences in the relative abundances of proteins, other strategies have been developed to quantify changes in the phosphorylation state of proteins, which has historically been performed using radionuclides. The phosphoprotein isotopecoded affinity tag (PhIAT) approach differentially labels phosphoseryl (pSer) and phosphothreonyl (pThr) residues with a stable isotopic and biotinylated tag, as shown in Figure 4.[5] The PhIAT strategy enriches the phosphoprotein pool in a manner that enables a quantitative measurement of phosphorylation to be made between the two distinct protein samples by comparing the extent of isotopic enrichment. After chemically blocking cysteinyl sulfhydryls via performic acid oxidation, phosphoproteins are selectively modified by removing the phosphate group from pSer and pThr residues via hydroxide ion mediated b-elimination. Michael addition of 1,2-ethanedithiol (EDT) to the newly formed α,β-unsaturated residues is performed using EDT containing either four alkyl hydrogens (EDT-D_0) or deuteriums (EDT-D_4) to achieve stable isotopic labeling. The sulfhydryl groups present on EDT-labeled proteins are biotinylated using iodoacetyl-PEO-biotin to generate PhIAT-labeled proteins. The PhIAT-labeled proteins are then digested with trypsin and isolated using immobilized avidin prior to LC/MS analysis. The result is the presence of two isotopically distinct versions of the same phosphopeptide allowing changes in the peptide's phosphorylation state to be quantified. Successful PhIAT labeling of a control phosphoprotein as well as proteins from a yeast extract was demonstrated.

The above presents only a glimpse into the several different types of stable isotope-labeling techniques that are being utilized in proteomics. As this field continues to develop, a continued growth in the variety and usefulness of stable isotope labeling methods will also develop. In particular, it is anticipated that the use of stable isotope-labeling methods to identify and quantify post-translational modifications will become an area of particular importance.

References

1. Pasa-Tolic, L.; Jensen P.K.; Anderson G.A.; Lipton, M.S.; Peden, K.K.; Martinovic. S.; Tolic. N.; Bruce, J.E.; Smith R,D. **1999**. High throughput proteome-wide measurements of protein expression using mass spectrometry. *J Am Chem Soc, 121,* 7949.

2. Oda, Y.; Huang, K.; Cross, F.R.; Crowburn, D.; Chait, B.T. **1999**. Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci USA, 96,* 6591.

3. Gygi, S.P.; Rist, B.; Gerber, S.A.; Turecek, F.; Gelb, M.H.; Aebersold, R. **1999**. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol, 17,* 994.

4. Conrads, T.P.; Alving, K.; Veenstra, T.D.; Belov, M.E.; Anderson, G.A.; Anderson, D.J.; Lipton, M.S.; Pasa-Tolic, L.; Udseth, H.R.; Chrisler, W.B.; Thrall, B.D.; Smith, R.D. **2001**. Quantitative analysis of bacterial and mammalian proteomes using a combination of cysteine affinity tags and ^{15}N-metabolic labeling. *Anal Chem,* in press.

5. Goshe, M.B.; Conrads, T.P.; Panisko, E.A.; Angell, N.H.; Veenstra, T.D.; Smith, R.D. **2001**. Phosphoprotein isotope-coded affinity tag (PhIAT) approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal Chem, 73,* 2578.

*Dr. Timothy Veenstra's current address is: NCI FRCDC/SAIC, Bldg. 469, Room 160, PO Box B, Fredrick, MD 21702 USA