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# In-Gel Stable-Isotope Labeling (ISIL): A Strategy for Mass Spectrometry-Based Relative Quantification

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Most proteomics approaches for relative quantification of protein expression use a combination of stable-isotope labeling and mass spectrometry. Traditionally, researchers have used difference gel electrophoresis (DIGE) from stained 1D and 2D gels for relative quantification. While differences in protein staining intensity can often be visualized, abundant proteins can obscure less abundant proteins, and quantification of post-translational modifications is difficult. A method is presented for quantifying changes in the abundance of a specific protein or changes in specific modifications of a protein using In-gel Stable-Isotope Labeling (ISIL). Proteins extracted from any source (tissue, cell line, immunoprecipitate, etc.), treated under two experimental conditions, are resolved in separate lanes by gel electrophoresis. The regions of interest (visualized by staining) are reacted separately with light versus heavy isotope-labeled reagents, and the gel slices are then mixed and digested with proteases. The resulting peptides are then analyzed by LC−MS to determine relative abundance of light/heavy isotope pairs and analyzed by LC−MS/MS for identification of sequence and modifications. The strategy compares well with other relative quantification strategies, and in silico calculations reveal its effectiveness as a global relative quantification strategy. An advantage of ISIL is that visualization of gel differences can be used as a first quantification step followed by accurate and sensitive protein level stable-isotope labeling and mass spectrometry-based relative quantification.

**Keywords:** Stable-isotope labeling • gel electrophoresis • relative quantification • mass spectrometry • LC−MS • proteomics • post-translational modification • phosphorylation • SILAC

## Introduction

Mass spectrometry has become the method of choice for many proteomics applications, including protein identification, mapping of post-translational modifications (PTM), and relative quantification of levels of protein expression and PTMs.[1−3] While a monumental goal of proteomics is the ability to globally probe an entire proteome, all of its post-translational modifications, and relative expression profiles in order to uncover insights into human disease and identify potential drug targets, proteomics has greatly excelled in targeted areas within a specific biological pathway through isolation and enrichment for specific proteins and protein complexes. For these proteomics applications, the final readout is often two lanes of a silver- or coomassie blue-stained one-dimensional (1D) SDS-PAGE gel for immunoprecipitations or a more complex two-dimensional (2D) polyacrylamide gel for whole cell lysates, and differences in protein expression or protein binding are typically visualized, commonly referred to as difference gel electrophoresis (DIGE).[4,5] If differences are observed, gel bands are excised, digested, and identified using tandem mass spectrometry and database searching.[1] While this approach works well in many cases, it fails to reveal proteins that change in level but are masked by highly abundant proteins in the same molecular weight range. Also, some post-translational modifications do not give rise to a band shift, and specific sites of modification cannot be determined. As a result of these limitations, proteomics researchers have generally turned to more elegant approaches of relative quantification based on stable-isotope labeling coupled with mass spectrometry as the readout, thus, avoiding gel-based methods.

The first major breakthrough in protein level isotope labeling approaches for proteomics was isotope-coded affinity tags (ICAT) introduced in 1999 by Gygi and Aebersold. In the ICAT approach,[6] cysteine residues of proteins from two different sources are labeled with light- and heavy-biotinylated reagents, respectively, mixed, and digested with trypsin. Cys-containing peptides are then enriched using an avidin column, and ratios of light-to-heavy peptide pairs analyzed by mass spectrometry

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directly reveal relative quantitative information. An advantage of this method is that only cysteine residues are labeled, therefore, simplifying the peptide mixture. The disadvantage is that not all proteins contain cysteine residues, and it is nearly impossible to get quantitative information on post-translational modifications unless the modified peptide contains cysteine residues.

For researchers growing cells, a popular approach is stable-isotope labeling of amino acids in cell culture (SILAC), a metabolic labeling method developed in 2002 by Ong and Mann.[7] In this approach, two sets of cells are grown independently with one set having heavy amino acids substituted into the culture media such as $^{13}C_6$-lysine and $^{13}C_6$-arginine. Cell lysates from different experimental conditions are then mixed and digested with trypsin. Subsequent analysis by mass spectrometry is performed to reveal relative quantitative differences. The approach has many advantages: isotope incorporation takes place during protein synthesis guaranteeing wide coverage, and by using isotopes of Lys and Arg, tryptic digestion results in isotope incorporation into all tryptic peptides. For those researchers working with yeast, $^{15}N$-labeled metabolic labeling with an ammonium source in the growing media alongside cells growing in $^{14}N$ media achieves global labeling.[8]

While several global postdigestion peptide level isotopic labeling approaches are popular, such as global internal standard technology (GIST),[9] isobaric tags for relative and absolute quantitation (iTRAQ) developed by Pappin in 2004,[10] and trypsin-catalyzed $^{18}O$ incorporation of peptides,[11] these approaches have the disadvantage that variability in extent of protease digestion of multiple samples treated in parallel can yield errors in quantification.[4]

In this article, we show that reagents previously developed for stable-isotope labeling of peptides can be used to label intact proteins in polyacrylamide gel matrixes and demonstrate that this approach provides global coverage and reproducible relative quantification of protein levels and protein modifications. In in-gel stable-isotope labeling (ISIL), proteins from two different experimental conditions are first separated using gel electrophoresis and separately labeled with stable isotopes in the gel matrix. The labeled proteins from the two conditions are then mixed together, digested, and analyzed by LC−MS. As an example of the strategy, we demonstrate how reagents for global internal standard technology (GIST), originally developed as a peptide level strategy by Ji and Regnier in 2000[9] can be applied at the protein level in gel sections for comparative proteomics analysis. This class of reagents was chosen since the reagents are inexpensive and easy to synthesize in most laboratories. In the typical GIST approach, primary amine groups (lysine residues and the N-terminus of peptides) are acetylated using succinimide-based reagents. At the protein level, lysine residues and the N-terminus of the protein are labeled. Schmidt et al. recently showed that similar succinimide-based reagents can be used effectively for protein level quantification.[12] However, in many cases, a protein's N-terminus is either blocked or not recovered in the LC−MS/MS experiment. Since most molecular and cellular biologists use gel electrophoresis as a common analytical method for visualizing the results of experiments, the ISIL strategy will give researchers an additional resource for accurate quantification of protein bands without the need for Western blotting, which is limited by availability of reliable antibodies. Importantly, it also allows researchers to quantify critical post-translational modifications.

## Experimental Methods

**Materials.** HPLC grade water, HPLC grade acetonitrile, and ammonium bicarbonate were purchased form Fisher Scientific (Pittsburgh, PA). Dithiothreitol, iodoacetamide, doubly distilled acetic acid, 50% hydroxylamine solution, acetic anhydride, $d_6$-acetic anhydride, *N*-hydroxysuccinimide, and HEPES were purchased from Sigma Chemical Co. (St. Louis, MO). Microcapillary columns were purchased from New Objective, Inc. (Woburn, MA). $C_{18}$ resin (5 $\mu$m, 100 Å) column packing material was purchased from Michrom Bioresources, Inc. (Auburn, CA).

**Gel Electrophoresis.** Standard SDS gels including fixed percentage and gradient polyacrylamide gels were run using both mini and large gel formats. Gels were run using standard methods and stained with standard protocols for mass spectrometry compatibility for both coomassie brilliant blue and silver stains.

**In-Gel Stable-Isotope Labeling and Enzymatic Digestions.** Gel slices from adjacent SDS-PAGE lanes were excised with meticulous care using a scalpel to ensure that identical gel volumes were used for both light and heavy isotope labeling reactions. Gel pieces were washed, cut into 1 mm$^3$ sections, and dried in a ThermoElectron SpeedVac concentrator. Cysteine residues of proteins were reduced with 10 $\mu$L of 10 mM DTT at 56 °C for 30 min and alkylated with 10 $\mu$L of 55 mM iodoacetamide for 45 min at room temperature. Gel pieces were then washed two times with 150 $\mu$L of 50% acetonitrile and dried in a SpeedVac. Approximately 1 mg of light *N*-acetoxy-($^1H_3$)succinimide and 1 mg of heavy *N*-acetoxy($^2H_3$)succinimide were added to the tubes containing the dried gel pieces, respectively (see ref 9 for reagent synthesis). Fifty microliters of 50 mM HEPES (pH = 8.3) was added to each tube, vortexed for 1 min to dissolve as much reagent as possible, and spun down in a microcentrifuge. Reactions were allowed to incubate for 3 h at room temperature. The gel slices were washed with 150 $\mu$L of 100mM ammonium bicarbonate followed by 20 $\mu$L of 50% hydroxylamine solution for 20 min to quench the reaction and reverse any side reactions, most notably tyrosine modifications. The combined gel slices were then washed three times with 150 $\mu$L of 100mM ammonium bicarbonate and one time with 50 $\mu$L of acetonitrile prior to drying in a SpeedVac. Twelve microliters of 25 ng/mL modified sequencing-grade trypsin−chymotrypsin in 50 mM ammonium bicarbonate was added to the combined gel pieces and placed on ice for 15 min to swell the gel pieces with enzyme solution. An additional 45 $\mu$L of 50 mM ammonium bicarbonate (pH = 8.4) was added and was incubated at 37 °C overnight. Digested peptides were extracted with 35 $\mu$L of 20 mM ammonium bicarbonate at 37 °C for 15 min, followed by 70 $\mu$L of 2% formic acid/40% acetonitrile at 37 °C for 15 min, and dried in a SpeedVac to a final volume of 10 $\mu$L.

**Microcapillary LC−MS/MS Analysis of Stable-Isotope-Labeled Peptide Pairs.** For protein identification and post-translational modification mapping, a 2 $\mu$L aliquot of digested peptides was run via microcapillary reversed-phase liquid chromatography tandem mass spectrometry using a self-packed 75 $\mu$m i.d. column self-packed with $C_{18}$ resin to 10 cm in length and a LCQ Deca ion trap mass spectrometer (ThermoElectron Corp., San Jose, CA) operated in positive ion LC−MS/MS mode. The HPLC gradient was 5% B to 40% B over 40 min, followed by a 5 min wash at 95% B and a 20 min postrun equilibration at 0% B (A, 99% water/0.9% acetonitrile/0.1% acetic acid; B, 99% acetonitrile/0.9% water/0.1% acetic acid) at a flow rate of 250 nL/min. Peptide sequences were identified

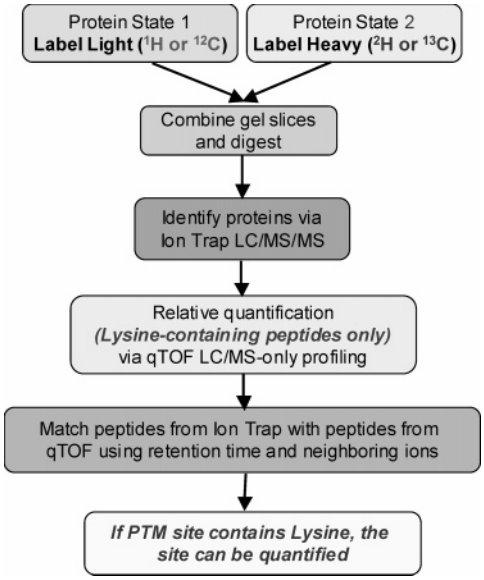


**Figure 1.** The workflow of the In-gel Stable-Isotope Labeling (ISIL) strategy for relative quantification of protein expression and/or post-translational modifications by microcapillary LC−MS and LC−MS/MS.

through a data-dependent LC−MS/MS acquisition. MS/MS spectra were searched against the PIR-NREF all species nonredundant protein database (http://pir.georgetown.edu/pirwww/search/searchseq.html) using the Sequest database searching algorithm with the following differential modifications for lysine residues: unmodified, modified with light reagent (+42.01 on K), and modified with heavy reagent (+45.01 on K) to be sure that all peptides selected for MS/MS analysis could be identified. After sequence and modification identification, ISIL peptide ion pair ratios were determined with an additional 2 $\mu$L aliquot of the same peptide digest using a high-resolution QSTAR Pulsar i hybrid quadrupole-TOF (qTOF) mass spectrometer (Applied Biosystems/Sciex, Framingham, MA) operated in positive ion LC−MS-only mode. Microcapillary columns as well as LC−MS buffers and gradient conditions were identical for both the LCQ and QSTAR systems. Ratios for lysine-containing peptide ions were calculated using automated GISTool software (see ref 13). Peptide ions were matched from both instruments by using similarities of neighboring ions, retention time, and charge state (see Figure 2).

## Results and Discussion

**Mass Spectrometry Strategy.** Gel slices from two adjacent gel lanes that contain proteins from two different experimental conditions were excised, isotopically labeled with light *N*-acetoxy-($^1H_3$)succinimide and heavy *N*-acetoxy($^2H_3$)succinimide GIST reagents, combined, and digested with a protease, and the resulting peptides were identified by LC−MS/MS and quantified by LC−MS according to the workflow in Figure 1.

We utilized a strategy whereby an ion trap mass spectrometer was used for identification of peptide sequences and post-translational modification sites due to fast scanning and fast duty cycle capabilities during data-dependent acquisitions. A qTOF mass spectrometer was then used for quantitative ratio determination of peptide ion pairs due to high-resolution and mass accuracy capabilities. On the basis of our experience with peptide mixtures, ion trap mass spectrometers usually produce slightly more sequence results than qTOF mass spectrometers, accounting for a slight increase in amino acid coverage per protein since more MS/MS data can be acquired per chromatographic time scale. For this reason, the ion trap was chosen to identify lysine-containing peptides, especially peptides containing post-translational modifications, since the modified peptides are usually present in lower amounts. However, the ion trap has relatively poor mass accuracy and resolution compared to the quadrupole-TOF instrument, so LC−MS-only runs using the qTOF were necessary to obtain accurate quantitative ratios of labeled peptide ion pairs. In our lab, the qTOF has a slight sensitivity edge over the ion trap in MS survey mode. Figure 2 shows the strategy for matching ions from two fundamentally different mass spectrometers, the ion trap and the qTOF. Note that the strategy for matching ions from two different instruments can be avoided with a high-end instrument such as the ion trap Fourier transform mass spectrometer (LTQ-FT) that combines the advantages of both instruments.[14] GISTool software, developed by Zhang et. al.[13] was used to accurately calculate the ratios of light-to-heavy-labeled peptide ions. GISTool has many features including de-isotoping, peak deconvolution, corrections for overlapping peptides, and a very successful correction for slight variations in retention time due to the deuterium effect.

**Labeling Efficiency and Accuracy.** Two SDS-PAGE gel pieces containing nearly identical protein amounts of the α and $\beta$ subunits of the phosphinositide-3-kinase (PI3K) regulatory protein (p85), immunoprecipitated from CHO cells, were labeled with light and heavy reagents, respectively, mixed, and digested together using trypsin. As an example of the data, Figure 3a shows the spectrum of the doubly charged tryptic peptide ion pair from p85 acquired by LC−MS via a quadrupole-TOF mass spectrometer for the sequence T**K**LEQDLR with light- and heavy-labeled peptide ions at $m/z$ 522.748 and $m/z$ 524.255 from each gel slice, respectively. Each label results in the addition of an acetate group to the lysine residue, +42 Da, or a deuterated acetate group, +45 Da (notice that acetylation of the Lys residue blocks tryptic digestion). Since one lysine residue is present in the peptide, only one acetate tag is incorporated into the peptide, which results in a 3 Da difference between the light- and heavy-labeled peptides. Since the doubly charged version of the peptide is observed, the $m/z$ difference appears as 1.5. Figure 3b shows the p85 tryptic peptide LRDTPDGTFLVR that contains no isotopic label since no lysine residues are present. For this peptide ion, a doubly charged singlet appears at $m/z$ 695.325, so relative quantification cannot be achieved.

Several approaches were used to test the labeling efficiency and accuracy of the ISIL strategy. First, data-dependent LC−MS/MS for the p85 digestion mixture was performed, and MS/MS spectra were searched against a nonredundant protein database for three possible forms of lysine states—light labeled, heavy labeled, and unlabeled, the latter representing incomplete modification in the experimental procedure. For the p85 experiment, 37 unique peptides were identified, 18 of which contained labeled Lys. Three of the 37 peptides had an unlabeled Lys, but these were less than 5% as abundant as the labeled form of the same Lys residues that also appeared. Since signals due to unlabeled Lys-containing peptides were much weaker compared to the labeled form of the same peptide, the overall quantification was not compromised by incomplete labeling. In no case was a lysine-containing peptide detected only in the unlabeled form. Figure 3c shows a graph of the peptide ion pair ratios calculated by GISTool software for the

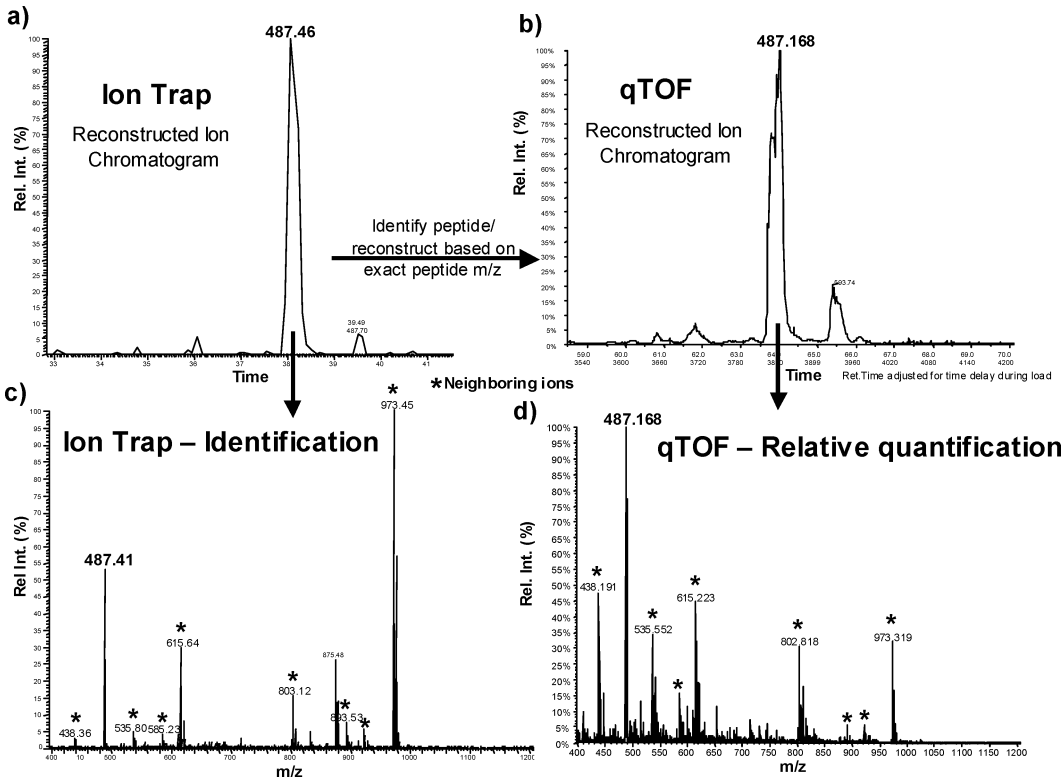


**Figure 2.** (a) The LC−MS/MS reconstructed ion chromatogram (RIC) of the light-labeled peptide ion at *m*/*z* 487.46 that is part of an ISIL doublet using the ion trap instrument. (b) The LC−MS reconstructed ion chromatogram for the same peptide ion at *m*/*z* 487.168 using the more accurate qTOF instrument. (c) The ion trap LC−MS-only spectra under the *m*/*z* 487 peak shows the ion of interest as well as neighboring peptide ion pairs and their respective charge states. (d) The same neighboring ions and charge states exist in the same chromatographic region of the *m*/*z* 487 peak from the qTOF instrument, which allows for the matching of the same peptide ion from different instruments.

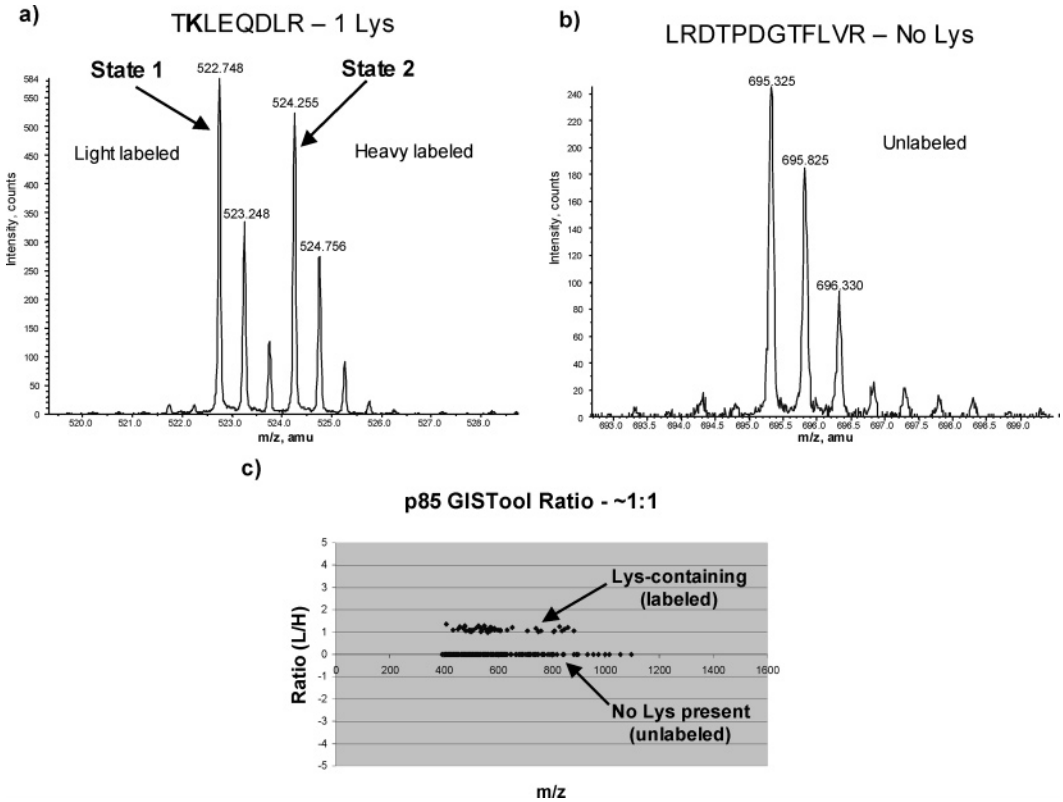


**Figure 3.** (a) An example of an ISIL-labeled peptide ion pair showing the light- and heavy-labeled peptide separated by 1.5 *m*/*z* units since the peptide is doubly charged and contains one labeled Lys residue (+3 Da). (b) An example of an unlabeled peptide showing a singlet ion, since the peptide does not contain a Lys residue. (c) The results of the GISTool software, which generates light/heavy ratios for all peptide ion pairs over the entire LC−MS experiment from a qTOF mass spectrometer. The peptide ion pairs that contain Lys residues result in a mean ratio of ~1.0 for equal amounts of a p85 digestion, while non-Lys-containing peptide ions are unlabeled singlets appearing at a ratio of 0.

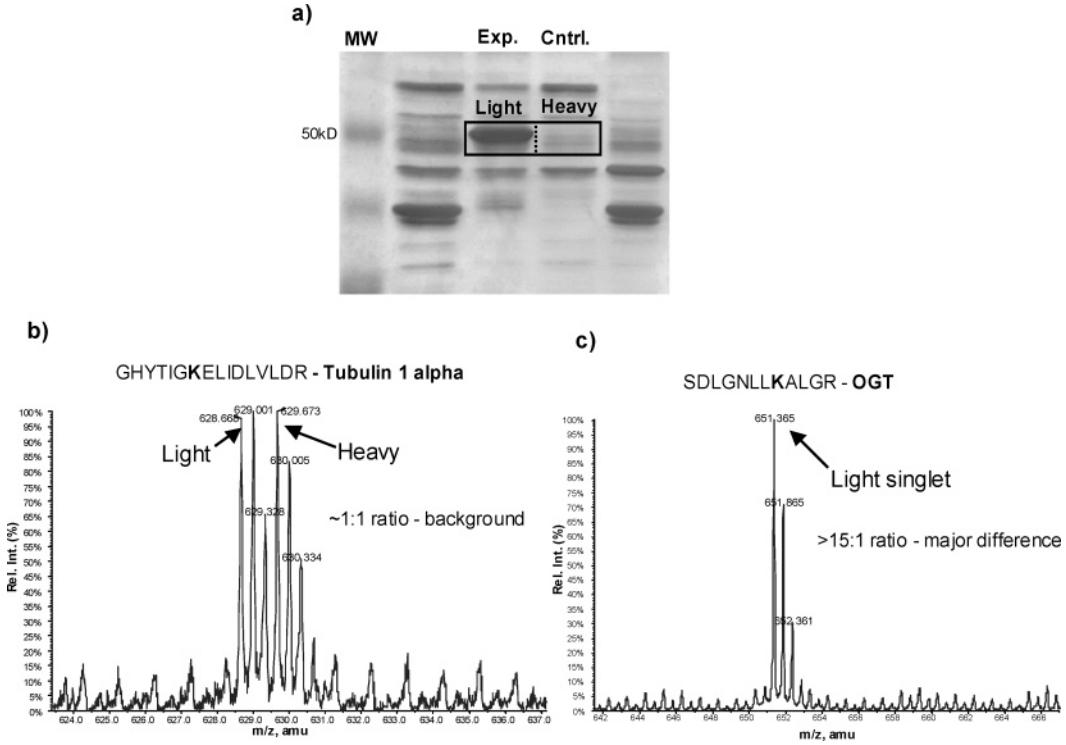


**Figure 4.** (a) A silver-stained SDS-PAGE gel containing the two adjacent regions that were excised for in-gel stable-isotope labeling. (b) An example of a low-level background protein that appears at a 1:1 ratio since it is present in equal quantities in both gel sections. (c) The major difference protein OGT that appears as a light-labeled singlet peak, the major difference peak observed from the gel.

p85 ISIL experiment. Lysine-containing peptides show a ~1:1 ratio of light-to-heavy label consistent with similar amounts of p85 in both lanes. For the 18 unique p85 labeled peptide ion pairs, the mean ratio and standard deviation was 1.13 ± 0.089, an indication of high experimental accuracy and reproducibility of ISIL among different peptides for a single protein. Nonlabeled peptides show a ratio of 0, indicating that no lysine is present. Some Lys-containing peptide ion pairs occur multiple times due to detection of multiple charge states in the electrospray experiment. By visual inspection of the raw data, all Lys-containing peptides appear to be properly labeled. For the doubly charged peptide ion pairs that were manually inspected, no peaks representing overlabeling at 21 $m/z$ units for light and 22.5 $m/z$ units for heavy (42 for the acetate and 45 for the deuterated acetate label divided by 2 for the charge state) greater than the peptide ion pair were observed. Similarly, no peaks representing underlabeling were observed.

**Determining Which Proteins Are Changing When Multiple Proteins Are Present in a Single Band.** A major advantage of this approach is that the presence of multiple proteins in a stained protein band is not a problem and can provide internal standards for normalization. The bands from the two lanes of the 50 kDa region of the gel in Figure 4a, indicated as Exp. and Cntrl., were labeled separately with light and heavy reagents, then combined, digested with trypsin, and analyzed by ion trap and then by qTOF mass spectrometers. Two major proteins were identified, Tubulin 1 α and *N*-acetylglucosaminyltransferase (OGT). The ratio of light-to-heavy Tubulin was 1:1 between Exp. and Cntrl. (Figure 4b), while the ratio of OGT was greater than 15:1, indicating that the difference in stain is due to changes in OGT rather than Tubulin (Figure 4c). The contaminating Tubulin can be used as a loading control.

**Comparison to SILAC for Identification and Quantification of pTyr Binding Proteins.** An experiment using stable-isotope labeling of amino acids in cell culture (SILAC) as a screen to identify SH2 and PTB binding domain proteins that bind to a phosphotyrosine peptide library (pTyr) yielded many results (manuscript in preparation). A pTyr library column was used to purify proteins from $^{13}$C-Arg, $^{13}$C-Lys-labeled HeLa cells, and a nonphosphorylated Tyr library column was used to purify proteins from $^{12}$C-Arg, $^{12}$C-Lys-labeled HeLa cells. The eluants from the two columns were combined prior to SDS-PAGE. Gel sections were excised, digested with trypsin, and analyzed by LC−MS/MS with a qTOF mass spectrometer. Proteins were identified using the Mascot database searching algorithm and MSQuant software (http://msquant.sourceforge.net/) developed by Matthias Mann for relative quantification. One protein identified by this approach was a phospho-Tyr phosphatase (PTPase), which appeared only as heavy-labeled singlet peptides since it did not significantly bind to the nonphosphorylated Tyr-column (Figure 5a). To reproduce the SILAC experiment using the ISIL strategy, pTyr and Tyr column elutions from unlabeled HeLa cells were run in separate lanes by SDS-PAGE as shown in Figure 5b. The experimental (pTyr) and control (Tyr) gel sections where the known pTyr binding protein, protein-tyrosine phosphatase 2C, migrated in the SILAC experiment were excised, labeled with light and heavy reagents, respectively, combined, and digested together with trypsin. An example of the results from one peptide of the same PTPase is shown in Figure 5c (light-labeled peptide ENVGLMQQQ**K**SFR at $m/z$ 812.384). No heavy-labeled version of the peptide was detected, indicating that it did not bind to the nonphosphorylated Tyr column. Notice that the ISIL results are in effect identical to the SILAC results for the same gel region, indicating preferential binding to only the pTyr peptide

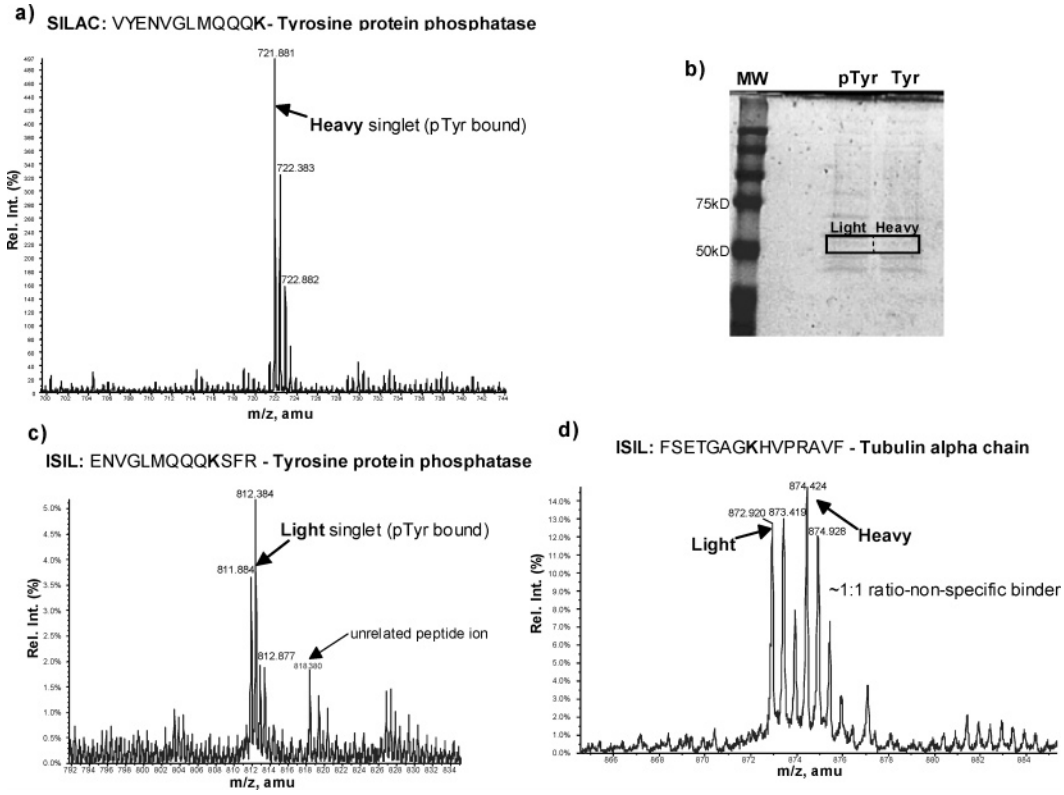


**Figure 5.** (a) The results from a SILAC experiment showing a heavy isotopically labeled singlet peak from a PTP-2C peptide. (b) A coomassie blue-stained SDS-PAGE gel showing two adjacent gel sections excised for relative quantification by ISIL. Notice that no differences can be observed by visualization. (c) The ISIL results showing the pTyr binding protein PTP-2C as a light-labeled singlet peak, demonstrating the selective binding to the phosphotyrosine column, essentially identical to ISIL results. (d) The nonspecific binding protein Tubulin is present at approximately at a 1:1 ratio as a background control.

library column. Figure 5d shows a doublet nontryptic peptide ion for Tubulin α chain at a ~1:1 ratio that was in the same region of the gel. These results demonstrate a situation where coomassie blue staining of the gel did not yield any visual differences, and the use of in-gel stable-isotope labeling followed by mass spectrometric analysis shows clear quantifiable differences in a specific protein. It also shows that the ISIL strategy can be as powerful in some cases as other protein level global quantification methods such as SILAC.

**Relative Quantification of Phosphorylation Sites.** An area of strong interest was to test the usefulness of the ISIL strategy for the accurate relative quantification of post-translational modification sites. For this experiment, the protein kinesin light chain 2 (KLC2) was immunoprecipitated under both normal and stimulating conditions. Figure 6a shows the experimental (heavy) and control (light) gel sections used for excision and in-gel stable-isotope labeling. A site of in vivo phosphorylation of KLC2 was previously determined in our lab; therefore, trypsin was used for proteolysis, since the sequence around the phosphorylation site predicted a peptide containing both a lysine and the phosphoserine residue of interest bracketed by Arg residues. Figure 6b shows the MS/MS spectrum of the light-labeled phosphopeptide SGpSFG**K**LR, confirming its identity. The MS/MS spectrum shows typical behavior of a phosphopeptide by tandem mass spectrometry with strong phosphoric acid ($H_3PO_4$) neutral loss fragment ions from the precursor ion and C-terminal fragments $y_6-y_8$.[15] Figure 6c shows the nonphosphorylated peptide pair with a ratio of 1.92, and Figure 6d shows the phosphorylated form of the same peptide with a ratio of 1.19 as calculated using the GISTool software. This is significantly different than the mean light/heavy ratio of 1.55 from the 29 other labeled peptides for KLC2 in this experiment, which is representative of the loading difference on the gel. The 25% decrease in the nonphosphopeptide signal in the Exp. lane likely represents an increase in the level of phosphorylation. To support this result, an identical 25% increase in phosphopeptide signal level in the Exp. sample was also observed. Note that two different phosphopeptides present in the sample did not change upon stimulation. This phosphopeptide example shows the utility of in-gel stable-isotope labeling for studying PTMs. This example also shows that corrections for unequal loading of lanes can be performed to reduce this source of error in assessing relatable phosphorylation stoichiometry.

**Effectiveness of Strategy and Peptide Coverage.** The effectiveness of a protein quantification method can be evaluated by many factors, such as sample complexity, technical difficulty, sensitivity, dynamic range, and average number of peptides that will be used for quantification. The ISIL strategy will have the greatest success for protein samples where complexity is reduced through chromatographic separation of proteins or immunoprecipitations of protein complexes prior to gel separation, therefore, reducing suppression effects. Technically, the method is simple, since it only adds several steps to standard in-gel digestion protocols and can be used with any gel-separated protein samples regardless of gel type or stain.

The dynamic range capabilities of the method are similar to other stable-isotope labeling methods that generate doublet peaks for MS-based quantification. For example, the maximum

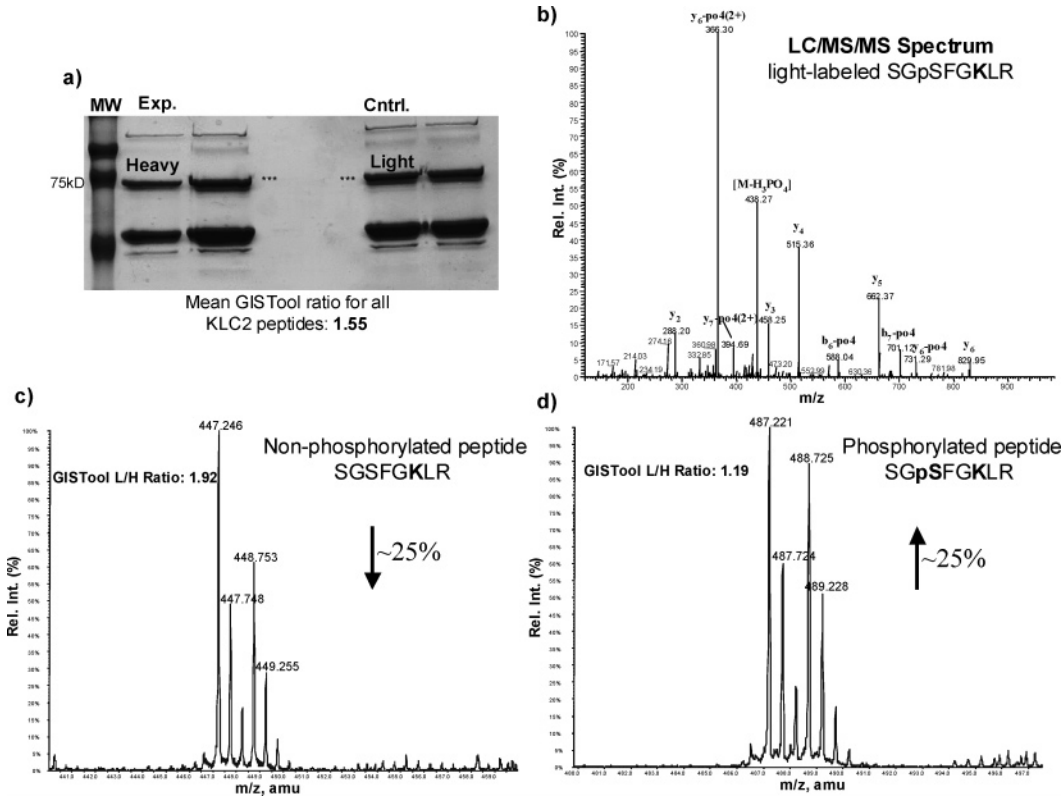


**Figure 6.** (a) The SDS-PAGE regions of the immunoprecipitated protein KLC2 under stimulated (Exp.) conditions and wild-type (Cntrl.) conditions. (b) The LC−MS/MS spectrum of the SGpSFG**K**LR phosphopeptide acquired using an ion trap mass spectrometer. (c) The LC−MS spectrum of the nonphosphorylated version of the peptide SGSFG**K**LR showing a decrease of ~25% in the stimulated sample compared to the mean ratio of all other KLC2 peptides. (d) The phosphorylated form of SGpSFG**K**LR shows an increase in signal of ~25% upon stimulating conditions compared to the mean ratio of all other labeled KLC2 peptides, supporting an increase in the level of phosphorylation.

ratio difference that can be detected using conventional mass spectrometers for the GIST, SILAC, ICAT, and ISIL methods is approximately 15:1. The dynamic range will increase with the use of a high-end instrument such as a FT−MS. In general, the sensitivity is equivalent to the sensitivity of typical in-gel digestions. In our hands, we can routinely identify and quantify low nanogram protein amounts from silver-stained gels.

For the proteins studied in our experiments using trypsin, peptide coverage is sufficient for accurate quantification, since approximately 40−50% of the proteolytic digestion products that were identified contained at least one labeled lysine residue. Trypsin cleaves at the C-terminus of Lys and Arg residues; however, labeled lysine residues prevent lysine cleavages resulting in only arginine cleavages. Trypsin essentially behaves like an ArgC protease in these experiments. Overall, this did not affect protein coverage, since very small peptides became larger for increased chromatographic retention and successful LC−MS/MS identification and few peptides became too large for analysis. Proteases that have multiple cleavage sites such as chymotrypsin resulted in less quantifiable peptides due to shorter peptide generation. In order for sites of post-translational modifications to be quantifiable, a modified peptide must also contain at least one lysine residue, and when trypsin is used, a PTM site located between two arginine residues will be missed. The fact that half of the peptides do not contain isotope labels could be viewed as a disadvantage of this strategy, especially for studying post-translational modifications. For these applications, it is usually best to first identify the PTM sites using standard proteomics methods and then choose an enzyme for ISIL that will result in the modified peptide containing a lysine residue.

**In Silico Comparison of ISIL, ICAT, GIST, and iTRAQ Methods.** In an effort to theoretically compare ISIL with existing post-protein expression isotope labeling methods, we performed an in silico simulation of ISIL, ICAT, and GIST/iTRAQ using protein sequence information downloaded from public databases. A single calculation was performed for both iTRAQ and GIST since the chemistry is very similar, resulting in essentially all peptides being labeled after digestion. *Escherichia coli* and yeast protein databases were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), while human and mouse protein databases were from IPI (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/). For the purposes of the simulation, we assumed complete enzymatic digestion, complete labeling, and each in silico peptide with molecular weight range from 500 to 6000 Da that liberally covers the entire experimentally detectable range for relative quantitative information for peptide mass spectra. However, a smaller molecular weight range is usually detected experimentally. It was also assumed that iodoacetamide and *N*-acetoxy succinimide reagents were used for both ISIL and GIST. Table 1 lists the possible protein coverage in terms of a percentage of the entire proteome for the ICAT, ISIL, and GIST/iTRAQ methods for the organisms *E. coli*, yeast, human, and mouse for the enzymes trypsin and chymotrypsin. The results show that the ISIL method can theoretically quantify more than 96% of proteins for all four organisms. The protein coverage of ISIL is higher than that for ICAT and is similar to that of the GIST/iTRAQ methods.

**Table 1.** The in Silico Predicted Protein Coverage from Quantifiable Peptides for Different Stable-Isotope Labeling Methods and Different Enzymes in a Global Proteomics Study[a]

| | organism | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *E. coli* | | Yeast | | Human | | Mouse | |
| enzyme | trypsin | chymotrypsin | trypsin | chymotrypsin | trypsin | chymotrypsin | trypsin | chymotrypsin |
| ICAT | 83.7% | 82.0% | 90.3% | 89.7% | 95.5% | 92.8% | 94.3% | 85.3% |
| ISIL | 97.2% | 98.2% | 97.5% | 99.3% | 96.5% | 96.1% | 96.4% | 95.9% |
| GIST/iTRAQ | 100.0% | 99.7% | 99.9% | 99.9% | 99.9% | 99.1% | 99.8% | 98.7% |

[a] Values given are a percentage of the entire proteome.

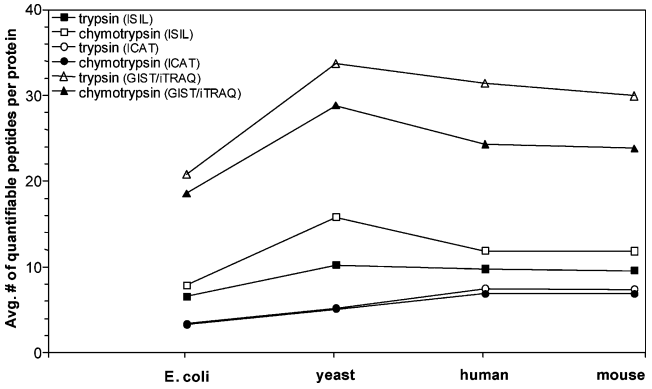


**Figure 7.** The average number of quantifiable peptides by LC−MS per protein and per organism using the ICAT, ISIL, and GIST/iTRAQ methods, assuming complete enzymatic digestion for peptides in the molecular weight range of 500−6000.

Experimentally, it is impossible that every in silico peptide can provide quantification information for a protein. Some of the in silico peptides will not be detected during the LC−MS experiment due to several factors including a peptide's ionization efficiency as well as protein concentration and the complexity of the peptide mixture. However, it is statistically true that more peptides will be experimentally detected from a group containing a large number of in silico peptides, compared with another group containing a smaller number of in silico peptides. Therefore, a large number of in silico peptides will provide more information for protein quantification. A peptide potentially containing quantitative information refers to a lysine-containing peptide for ISIL, a cysteine-containing peptide for ICAT, or any peptide for GIST/iTRAQ. We refer to these as detectable peptides. Figure 7 displays the average number of peptides per protein from different organisms. There are approximately 8∼10 peptides per proteins that carry quantitative information for ISIL, while this number is decreased to 3∼6 for ICAT and increased to 20∼32 for GIST and iTRAQ.

## Conclusions

Based on the collection of information including in silico results as well as our experimental results, we believe that the protein coverage for relative quantification by ISIL is sufficient for most proteomics studies. The ability to incorporate stable-isotope labels in-gel at the protein level after visualization of biological experiments with gel electrophoresis adds a new dimension for the relative quantification of protein expression and many post-translational modifications. Protein level labeling can be advantageous, since the isotope-labeled proteins are digested together avoiding any potential errors due to digestion irreproducibility. Any organism or protein source such as tissue can be used with ISIL. Since labels are incorporated after gel electrophoresis has been performed and since gel electrophoresis is used for the labeling step, further dimensions of separation to reduce sample complexity are usually unnecessary other than subsequent reversed-phase LC−MS. The strategy can be especially useful for serendipitous results from gel readouts, for uncovering differences that are masked by more abundant bands, and in many cases for post-translational modifications depending upon the labeling reagents selected and the enzyme used for digestion. The labeling process is efficient and can likely be used with most any labeling reagents such as the ICAT, commercially available iTRAQ, and other $^{13}C$ -based, succinimide-based reagents.[16] The method is also expected to work with all types of polyacrylamide gels regardless of percentage and including two-dimensional gels.

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## References

(1) Aebersold, R.; Mann, M. Mass spectrometry-based proteomics. *Nature* **2003**, *422*, 198−207.
(2) Mann, M.; Jensen, O. N. Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* **2003**, *21*, 255−261.
(3) Lill, J. Proteomic tools for quantitation by mass spectrometry. *Mass Spectrom. Rev.* **2003**, *22*, 182−194.
(4) Lilley, K. S.; Friedman, D. B. All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Rev. Proteomics* **2004**, *4*, 401−409.
(5) Kolkman, A.; Dirksen, E. H. C.; Slijper, M.; Heck, A. J. R. Double standards in quantitative proteomics—Direct comparative assessment of difference in gel electrophoresis and metabolic stable isotope labeling. *Mol. Cell. Proteomics* **2005**, *3*, 255−266.
(6) Gygi, S. O.; Rist, B.; Gerber, S. A.; Turacek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17*, 994−999.
(7) Ong, S. E.; Blagoev, B.; Kratchmarova, I.; Kristensen, D. B.; Steen, H.; Pandey A.; Mann, M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **2002**, *1*, 376−386.
(8) Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. *Anal. Chem.* **2002**, *74*, 1650−1657.
(9) Ji, J.; Chakraborty, A.; Geng M.; Zhang, X.; Amini, A.; Bina, M.; Regnier, F. E. Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. *J. Chromatogr., A* **2000**, 745, 197−210.
(10) Ross, P. L.; Huang, Y. L. N.; Marchese, J. N.; Pappin, D. J. C. Multiplexed protein quantification in *Saccharomyces cerevisiae*

using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics.* **2004**, *3*, 1154−1169.
(11) Mirgorodskaya, O. A.; Kozmin, Y. P.; Titov, M. I.; Körner, R.; Sönksen, C. P.; Roepstorff, P. Quantification of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using $^{18}$O-labeled internal standards. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 1226−1232.
(12) Schmidt, A.; Kellermann, J.; Lottspeich, F. A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics* **2005**, *5*, 4−15.
(13) Zhang, X.; Hines, W.; Adamec, J.; Asara, J. M.; Naylor, S.; Regnier, F. E. An automated method for the analysis of stable isotope labeling data in proteomics. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1181−1191.
(14) Ballif, B. A.; Roux, P. P.; Gerber, S. A.; MacKeigan, J. P.; Blenis, J.; Gygi, S. P. Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 667−672
(15) Resing, K. A.; Johnson, R. S.; Walsh, K. A. Mass-spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament-associated protein. *Biochemistry* **1995**, *34*, 9477−9487.
(16) Zhang, R. J.; Regnier, F. E. Minimizing resolution of isotopically coded peptides in comparative proteomics. *J. Proteome Res.* **2002**, *1*, 139−147.

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