Chapter 7

Multiplexed Isobaric Tagging Protocols for Quantitative Mass Spectrometry Approaches to Auditory Research

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Abstract

Modern biologists have at their disposal a large array of techniques used to assess the existence and relative or absolute quantity of any molecule of interest in a sample. However, implementing most of these procedures can be a daunting task for the first time, even in a lab with experienced researchers. Just choosing a protocol to follow can take weeks while all of the nuances are examined and it is determined whether a protocol will (a) give the desired results, (b) result in interpretable and unbiased data, and (c) be amenable to the sample of interest. We detail here a robust procedure for labeling proteins in a complex lysate for the ultimate differential quantification of protein abundance following experimental manipulations. Following a successful outcome of the labeling procedure, the sample is submitted for mass spectrometric analysis, resulting in peptide quantification and protein identification. While we will concentrate on cells in culture, we will point out procedures that can be used for labeling lysates generated from tissues, along with any minor modifications required for such samples. We will also outline, but not fully document, other strategies used in our lab to label proteins prior to mass spectrometric analysis, and describe under which conditions each procedure may be desirable. What is not covered in this chapter is anything but the most brief introduction to mass spectrometry (instrumentation, theory, etc.), nor do we attempt to cover much in the way of software used for post hoc analysis. These two topics are dependent upon one's resources, and where applicable, one's collaborators. We strongly encourage the reader to seek out expert advice on topics not covered here.

Key words Proteomics, iTRAQ, Quantitative mass spectrometry, Protein expression

1 Introduction

A unique watershed moment in biology occurred with the release of the initial drafts of the entire genome sequence derived from first model organisms. This event led to the completion of whole genomic sequencing of many other organisms, including human. The ability to read genomic sequences and map genes within genomic space is certainly a powerful tool. However, it can also be argued that the vast majority of practicing biologists today actually work on understanding the role of *proteins* in their chosen sub-discipline, whether to seek an understanding of development,

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disease states and their progression, or normal cellular processes. Yet the methods used by many biologists to examine the transcriptome only details the state of the transcript and hence the state of gene expression; whereas, it is the protein(s) encoded in each transcript that is more often the actual effector molecule of interest. One assumption tacitly made is that gene expression equals protein expression, but this is clearly not always (some may say rarely!) true [1, 2]. Thus, as we push beyond the genomic age, into a post-genomic epoch of biology, one must increasingly come to terms with techniques that are more suited to assess the effectors of biological processes. The transcriptome is a static entity in terms of sequence (save for the uncommon, or experimentally induced, mutation and methylation), whereas proteins have many more degrees of freedom that make their assessment particularly challenging. These can include post-translational modifications, which can be many (phosphorylation, palmitoylation, sumolyation, etc.), the timing of these changes, alternative splicing from the genome, and RNA editing-induced changes to the protein sequence (and therefore function), which is not even revealed in the genomic sequence. Coupling these issues with the observation that many genes can encode more than one protein (and in some cases hundreds of proteins), one begins to get the sense of the enormous task facing the biologist wishing to examine the state of the proteome in their sample of interest. Indeed, while estimates of the number of genes making up the genome generally are settled between 20,000 and 30,000 genes, estimates of the functional proteome range is in the 100,000s. While biological complexity certainly resides within the genome, a simple comparison of the number and sequence of genes between the worm, *C. elegans*, and humans, illustrates that a major portion of the biological complexity separating these species resides in gene function, and even more importantly, in the interactions between gene products.

The study of proteins in biological processes crossed a watershed point with the coupling of mass spectrometry (MS) with biological samples, even though techniques used for assessing protein expression and quantification were in use for many years before. The "need" that led to this change in approach is traced to the difficulty of assessing large numbers of proteins simultaneously, as well as assessing proteins for which no discriminatory tag is available (either an antibody, or a fusion protein expressed in vivo). The chief advance made possible by MS-based proteomics is the freedom to discover otherwise unanticipated changes in protein expression, post-translational modification, etc., that are involved in particular biological processes. The evaluation of these expression states leads to the establishment of protein interaction networks. Many methods exist for establishing and quantifying interaction networks, however, they all result in testable hypotheses of mechanism(s) that regulate phenotypic changes in an organism.

The end result can reveal protein interactions not previously appreciated, as well as altered cellular states observed in disease or experimenter-induced changes in cells/tissues. Thus, an understanding can be attained of how a phenotype occurs when that phenotype is not directly related to an altered gene [3, 4]. Inherent in this kind of approach is the movement away from a purely reductionist view of biology, to a more global, interactive view characterized by the systems biology approach.

Many of the most successful proteomics experiments to date (at least related to higher organisms) have used proteomic approaches to study changes in specific structures such as organelles [5–7], synaptic junctional preparations [8], etc. This illustrates a key principle in proteomics—simplifying the sample results in better discrimination of protein changes.

There are a number of issues that must be considered once a decision is made to tackle a proteomics project. A firm commitment must be made to the type of data collection, since in most cases, strategies adopted may be mutually exclusive for other purposes. Thus, if there is an interest in examining phosphorylation states of proteins, a sample preparation/isolation method is used that is significantly different from more routine protein expression studies. Similarly, if interested in glycosylation states of membrane-associated proteins, isolation may be significantly different than when assessing the nuclear proteome. Other concerns include whether the sample is pre-fractionated prior to further processing, run on a one- or two-dimensional gel or a liquid chromatography column, tagged for future quantification, or whether the protein remains tagless. One rule of thumb is that each time the sample is manipulated there is a likelihood that protein loss *will* occur. The threshold at which these loses might alter the outcome of the experiment is difficult or impossible to know *a priori*.

Finally, although space does not allow a full description of mass spectrometry-based proteomics here, several review articles can help the uninitiated to navigate the decisions that must be made and to allow a better appreciation of the concerns [9–13]. Here, we limit ourselves to detailing the preparation of a protein lysate sample for quantifying expression levels in vitro and for giving a quick introduction to the analyses of the resulting data.

1.1 Labeling Methods for Quantitative Differential Mass Spectrometry

We will outline three of the more common methods of differential labeling in this section and detail one of them further in Section 3. Figure 1 schematically demonstrates the major steps of these methods side by side.

1.1.1 Isotope-Coded Affinity Tag (ICAT)

ICAT (Fig. 1a) was one of the first methods by which peptides could be differentially labeled prior to submission for mass spectrometric analysis [14]. Briefly, this technique uses a biotinylated reagent with a specificity toward sulfhydryl groups. The labeling

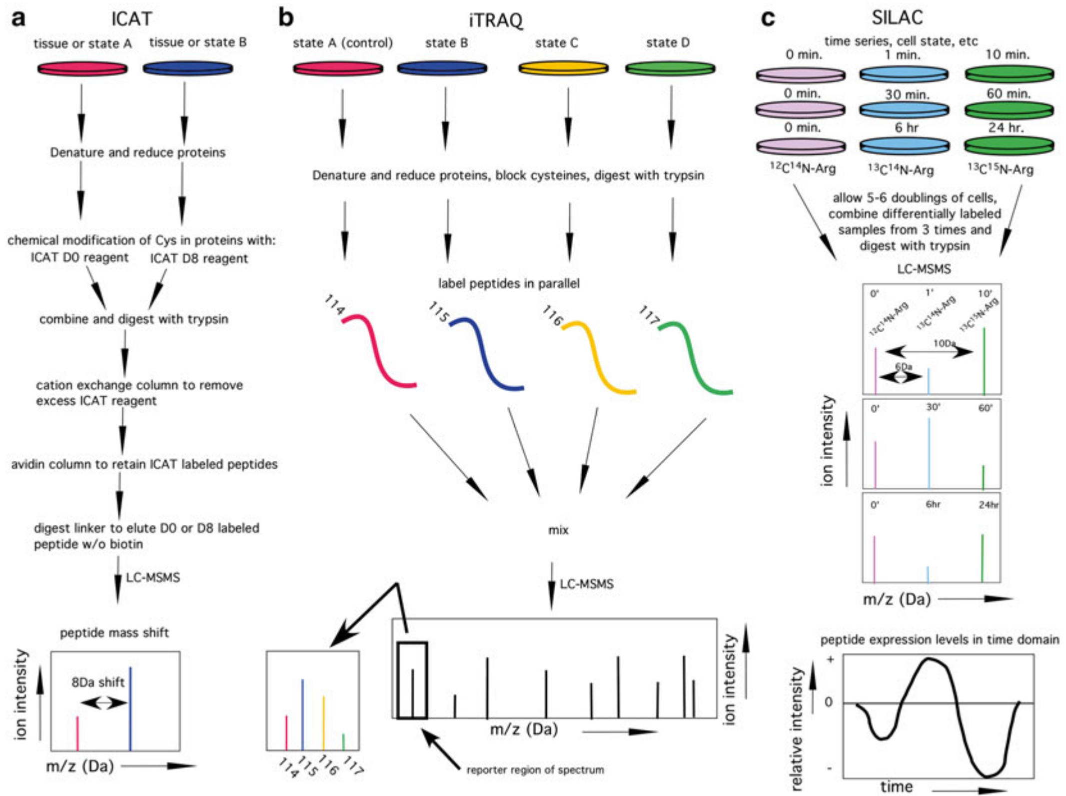

Fig. 1 Comparison of major steps in labeling proteins or peptides for quantitative mass spectrometric analysis. (**a**) When using ICAT, compare tissues or cells by first denaturing the proteins in the lysate and then labeling all cysteine residues with one of two labels. The light label contains normal hydrogen at specific sites, while the heavy label contains deuterium substituted in place of the hydrogens. In all, the heavy label is 8 Da heavier than the light label. Lysates are mixed at this point and digested with trypsin. Following digestion, the resultant-labeled peptides are selected for and retained over an affinity column, eluted and subjected to liquid chromatography and mass spectrometry. Peptides exactly 8 Da apart are examined and, when matched by sequence, the ion intensity (or the precursor LC peak area) is assessed for quantitation purposes. (**b**) When using iTRAQ, cells or tissues of different states are lysed, denatured, and digested. Each pool is labeled separately, then mixed, submitted to LC-MSMS, and each label is assessed for ion intensity. Labels are freed during peptide bond cleavage, and are found in a low mass region of the spectrum, separated by 1 Da. (**c**) In SILAC procedures, one metabolically labels all proteins by allowing the cells to incorporate various heavy-labeled arginine (depicted here) and/or lysine into the generated proteins. Lysates are obtained, mixed, digested, and any post-translational modifications desired are selected and submitted to LC-MSMS. Expression data are plotted in the time domain to gain an understanding of the temporal nature of modifications. For example, if one is looking at phosphorylation events, data can indicate the time course of phosphorylation and dephosphorylation, as depicted in the bottom panel

reagent carries either a “heavy” (d_8) or “light” (d_0) tag (Fig. 2a). This sample is combined with a second, independently labeled sample carrying the other tag, which is then compared to the first. The combined samples are then trypsinized. Labeled peptides are recovered from the mixture via biotin affinity chromatography.

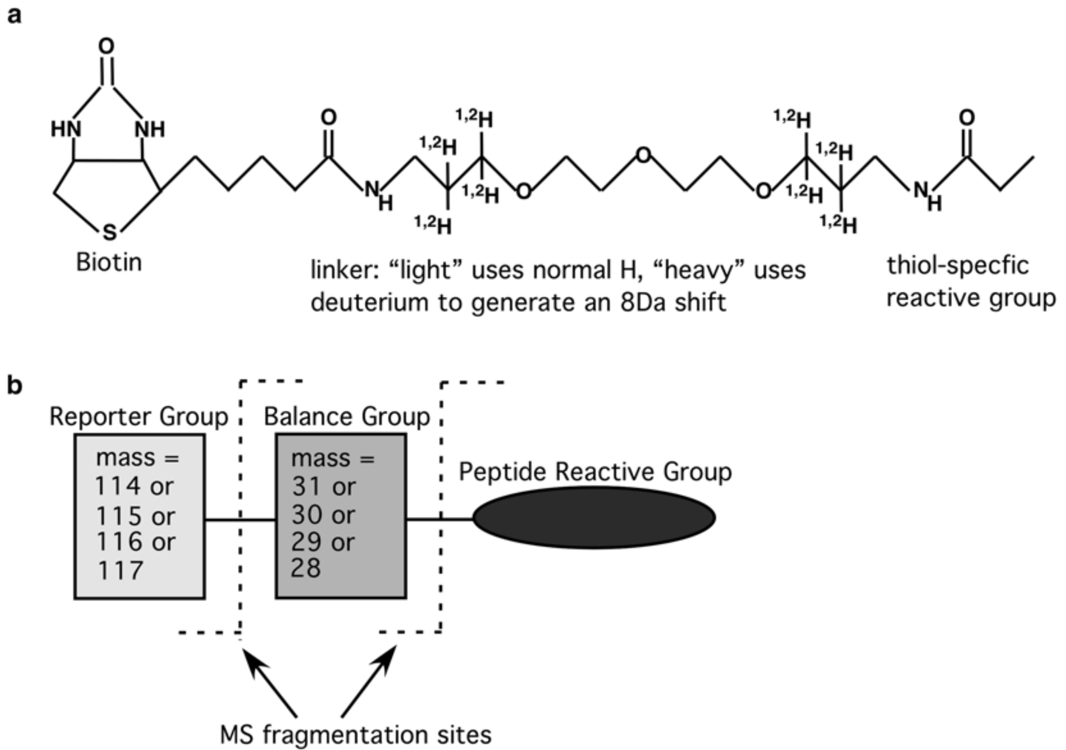

Fig. 2 Labels for ICAT and iTRAQ technologies. (**a**) The ICAT label is composed of a biotin moiety used for affinity selection, a linker, which contains either ^{1}H or D (^{2}H) substitutions as indicated, and a thiol reactive group that binds to free cysteines of the protein. The biotin group is cleaved after the affinity column selection step and the peptide released. (**b**) The iTRAQ label consists of a reporter group of varying mass and a balance group that is co-varied with the mass of the reporter group, such that each peptide labeled carries an isobaric tag (i.e. a tag of the same mass). The label also contains a peptide reactive group that is reactive toward primary amines and ε amines of lysine residues. Upon dissociation in the mass spectrometer, peptide bonds are broken, releasing the peptide and the balance and reporter groups. The peptide undergoes further peptide bond breaks to yield the MSMS peptide sequence, while the balance group is lost in the very low mass region of the spectrum. The reporter group is found in a very quiet region of the spectrum and is assessed for total ion count/intensity to yield the quantitative information needed for analysis

Following cleavage of the sample from the biotin moiety and release from the affinity column, the mixture is analyzed via liquid chromatography coupled mass spectrometry (LCMS). Analysis consists of examining the ratio of ion intensities of the heavy and light sequence-matched peptides in the MS. The final result yields both sequence information of the peptide following tandem MS procedures and quantification of each peptide. Two potential drawbacks of the ICAT approach are its dependence on the occurrence of cysteines in the proteins of interest (a minor difficulty, as most proteins do contain cysteine residues) and the number of cysteine residues. The latter can be more problematic if the proteins of interest contain few cysteine residues. Unambiguous protein

identification from a complex peptide mixture requires good coverage of the parent protein by the recovered peptides. Therefore, if there are few cysteine residues, and thus few labeled peptides, protein identification may be compromised. The advantage of the ICAT system is its relative ease of use, and its applicability to lysates generated from any source (cell culture, tissues, etc.). A number of protocols have been published concerning ICAT [15].

1.1.2 Isobaric Tagging for Relative and Absolute Quantification (iTRAQ)

iTRAQ (Fig. 1b) is similar in concept to ICAT, but differs in that amines are modified to carry a label for quantification (Fig. 2b). With iTRAQ, one also has the ability to multiplex up to four samples for simultaneous differential MS quantification. Unlike the ICAT system, with iTRAQ, samples are trypsinized first prior to labeling. This approach has the advantage of labeling all peptides, since trypsin cuts at lysine/arginine sequences, and the iTRAQ system labels ε amines of each lysine. In addition, other free amines carry a label as well. The manner by which quantification is accomplished in the MS is similar to the way ICAT ratios of ion intensities are used to assess changes in peptide expression between samples. In the mass spectra, the location of the quantified peaks is remote from the peptide sequence peaks, due to the cleavage of the label from the peptide during the collision-induced dissociation phase of the tandem MS run. The advantage here is that all label peaks at mass 114, 115, 116, and 117 are collected in one place in the spectrum that is relatively "quiet" (Fig. 4). Thus, suppression by abundant peptide species is generally very low, and the signal more accurately reflects the true expression level. An added advantage to iTRAQ over ICAT is that peptide coverage of protein sequences is more complete due to the greater number of labeled peptides available for analysis. This coverage leads to a better resolution of protein identification, especially with family members that have a highly conserved sequence. While iTRAQ can be used for samples generated from tissue as well as for cell culture, the majority of work thus far is from in vitro preparations.

1.1.3 Tandem Mass Tags (TMTs)

Labeling techniques for relative quantification of proteins have continued to evolve. The iTRAQ technique has continued to grow in popularity since the original publication of this volume. At the time of writing this update, over 1500 publications can be found on PubMed using iTRAQ as a search term. Perhaps one of the more significant advances in quantitative proteomics has been the continued evolution of instrumentation, but not to be overlooked are the developments occurring in the procedures of peptide labeling. Here we wish to highlight, in abbreviated form, the availability of a new set of mass tags that function very much like the iTRAQ reagents, but which also have some expanded properties that the end user may appreciate and find useful for specific experimental work flows. Thermo Scientific has developed a set of novel reagents

called tandem mass tags (TMTs) for use in peptide labeling [16]. Similar to the standard iTRAQ reagents, the TMT reagents are composed of an amide-reactive NHS-ester group, a spacer arm, and a mass reporter. The mass reporter is cleaved during MSMS, leaving resides in the 126–131 Da region of the MSMS spectrum. The standard kit allows six-plex labeling of complex samples, thus increasing the experimental design toward simultaneous analysis of six different conditions/time points, etc. Using appropriately equipped MS instruments capable of high-energy collision dissociation (HCD), one may take advantage of a 10-plex reagent kit. The 10-plex makes use of differential resolution of ^{13}C and ^{15}N isotopes within the mass reporter, which yield slightly different masses in the MSMS spectrum. For example, the TMT^{10}-127N will be visible with a monoisotopic reporter mass of 127.124760, while the same label carrying heavy carbon instead of nitrogen, termed TMT^{10}-127C, will have a mass of 127.131079.

Of special interest to investigators wishing to examine peptides potentially involved in disulfide bonds in the parent protein, an iodoacetyl TMT reagent has also become available from Thermo Scientific in either a single label or six-plex version. These kits employ a TMT tag containing a cys-reactive group that allows for quantitative investigations of cysteine containing peptides. The tag produces an irreversible labeling of sulfhydryl groups. Because of the presumed lower abundance of such peptides in complex samples, it is suggested that the investigator use an immobilized anti-TMT antibody resin to enrich the final submitted sample for the label. The analysis makes use of standard CID energies for MSMS, although HCD and ETD may also be used.

Finally, another specialty labeling kit produced by Thermo Scientific is the aminoxyTMT kit. This chemistry is useful for quantitative assessments of carbonyl-containing compounds and also comes as a single or six-plex labeling kit. The aminoxy group of the labeling reagent is reactive toward carbonyls. This is predicted to be useful for steroids, oxidized proteins, and especially carbohydrates. The kit is therefore of use for investigating the glycome via analysis of N-linked glycans. Unlike all of the labeling reagents considered to this point, the aminoxyTMT mass tag reagents are used following PNGaseF/G glycosidase treatment of samples (tissue/cells, fluids), which releases N-linked glycans. The N-linked glycans are then separated/enriched from the rest of the material via a simple hydrophobic column, and then labeled with the aminoxyTMT reagent of choice. The labeling occurs at the reducing end of the glycan. Given the complexity of the glycome, and the increasing interest in general glycobiology and variations of the glycome associated with disease states (for example, Huntington's Disease [17], etc.), the aminoxyTMT labeling kit may be very useful in proteome discovery pipelines.

1.2 Absolute Quantification Without Labeling

The techniques described above produce relative assessments of protein expression. They are all based on some basal level of expression from which a change under experimental manipulation is assessed. These analyses are most often pursued using a global discovery approach in which an information-dependent acquisition (IDA) of data is performed and matched across the global proteomic database. In an IDA approach, peptides are picked by the mass spectrometer for analysis based on several user-defined *a priori* rules, but which are generally designed to simply examine the highest peak intensities without a hypothesis driven reason. Additionally, all fragments (transitions) generated from that peptide are passed on for detection. While powerful in discovering the "lay of the land," it is a well-known problem of such MS approaches that results are too often impossible to fully replicate not only between different labs, but even in the same lab between runs. Potentially interesting peptides are not always observed in all sample replicates due to many factors, including sample complexity and the automated procedure of peak picking for MSMS analysis used in peptide identification. Sample complexity can result in lost identifications due to competition between co-eluting peptides (ion suppression), or simply from the mass spec being busy analyzing one signal while another is present in the chromatography run whose peak is lost (i.e. when the time differential between peaks is too short). To overcome such problems, a *m*ultiple *r*eaction *m*onitoring (MRM) MS technique can be developed (*see* [18] for review). MRM uses information based on first round discovery-mode (IDA) results to develop a highly specific *targeted-mode* approach to peptide tracking and quantification. MRM-MS tracks single transitions from predefined peptides of interest (i.e. those observed as differentially expressed under IDA mode). Because definitions of transition states are physical constants that are used to follow a peptide, results are highly reproducible between experiments and are obtained with highest sensitivity since only this transition is monitored at any time. Thus, MRM transition definitions also allow for independent follow-up of the peptide across labs. The key to biologically relevant and successful MRM studies lies in knowledge first gained during discovery phase (IDA) work, and depends on a previously complete analysis of proteins expressed in the sample under specific conditions. While in silico assumptions may be made concerning proteins and their transition states, this is no substitute for empirically derived data-driven MRM production. The largest challenge to adopting MRM as a standard practice for quantitative proteomics is the issue surrounding the time and cost of assay development. One must first select individual peptides for analysis, and this is required for numerous (dozens to hundreds) proteins. Absolute quantification is attained by producing stable isotope-labeled peptides that are distinguishable from native peptides assayed from the sample. These labeled peptides are

spiked into the mix and used as exemplars against which to compare the target peptide. Previous calibration of MS signal by known concentrations of each labeled peptide is used to determine the concentration of the targeted peptide, when the signal from the targeted peptide is compared to the labeled peptide spiked into the mix. The production of these calibration peptides can be expensive, given the requirement for purity and the use of stable isotopes in their production. Nonetheless, because of the selected monitoring of very specific transitions (fragmentations), MRM is extremely sensitive and highly reproducible. As MRM transition databases continue to grow, thus obviating the need for extensive early phase IDA analysis (and thereby decreasing some expense), MRM-based quantification will become the typical method for quantification of the proteome.

1.3 Stable Isotope Labeling of Amino Acids (SILAC) in Cell Culture

SILAC (Fig. 1c) is significantly different from either ICAT or iTRAQ style approaches. With the SILAC approach, all proteins are labeled *metabolically* via incorporation of labeled arginine, lysine, or both [19, 20]. Additionally, the label can be light, intermediate, or heavy by using the desired amino acid carrying ^{13}C, ^{15}N, or double $^{13}C^{15}N$ substitutions. Because of the number of possible labels (four if one also considers the unlabeled control state), multiplexing is possible with SILAC. The key to successful SILAC labeling is allowing the cells to completely incorporate the labeled amino acids into all the proteins. Therefore, cells are passaged a minimum of five to six times in the presence of the labeled amino acids to accomplish full incorporation [20]. SILAC techniques are used to create dynamic temporal maps of protein expression changes in response to various manipulations [21]. The strength of the SILAC approach resides in its tagless approach to differential MS analysis, thus alleviating potential LC problems (co-elution issues, etc.). Additionally, the population of cells analyzed is typically homogeneous, thereby simplifying the proteomic complexity normally inherent in tissue. Thus, signals are cleaner, allowing a deeper probe into the proteome and, thereby allowing access to less abundant and potentially more "biologically significant" proteins. The significant downside to SILAC is its inability to be used in complex organisms, although reports exist of metabolically labeling *Drosophilia* and *C. elegans* for quantitative proteomic analysis [22]. Additionally, a potential arginine to proline inter-conversion can take place if the arginine is not kept at sufficiently low concentrations in the culture media. This inter-conversion can lead to artifactual loss of arginine signal. However, the most likely problem faced when using SILAC techniques is the issue of cost. Stable isotopes of amino acids can be very expensive and specialized cell culture media is required. Also, serum additives generally cannot be used due to the potential for introducing unlabeled amino acids to the cells. Not all cells grow and thrive in serum-free conditions.

Numerous other methods exist [23] for labeling, such as ^{18}O labeling, and peptide acylation, more commonly referred to as a global internal standard technique (GIST). However, ICAT, iTRAQ, and SILAC represent the most commonly used techniques, and it is relatively straightforward to perform the labeling chemistry, as these are available in kit form from vendors.

2 Materials

2.1 Hardware

1. Refrigerated bench-top centrifuge capable of 18–20,000 × *g*.
2. SpeedVac® or similar vacuum concentrator.
3. 60 °C Heat block.
4. 37 °C Incubator.
5. Vortexer.
6. pH paper.
7. 2.5 mL Hamilton syringe with a blunt 22-gauge needle.
8. Standard lab pipettors.
9. Tissue grinder with ground glass surfaces (Kontes, Vineland, NJ).
10. PepClean C18 columns (Pierce, Milwaukee, WI).
11. Probe sonicator.
12. More sophisticated equipment may be required depending on the level of sample pre-fractionation desired, including Mini-Rotofor® (BioRad, Hercules, CA), FPLC, or HPLC capable of performing reverse phase chromatography. These more specialized pieces of equipment will not be covered further, but may be mentioned where appropriate.

2.2 Specialized Reagents/Solutions

2.2.1 Lysate Generation

1. Hanks' buffered saline solution (HBSS) without $CaCl_2$, $MgCl_2$, $MgSO_4$.
2. RIPA lysis buffer or a similar lysis solution such as Pierce's T-Per: 1 % NP-40 or Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2.
3. Protein assay kit.

2.2.2 iTRAQ Labeling

1. iTRAQ labeling system kit (Applied Biosystems, Foster City, CA).
2. Acetonitrile, high purity, stored at room temperature.
3. 0.5 % fresh trifluoroacetic acid in water preferably made from stock TFA packaged in glass ampoules and stored at room temperature.
4. Methanol, high quality, such as HPLC grade and stored at room temperature.

5. Absolute ethanol, high quality, such as HPLC grade, and stored at room temperature.
6. 2 % SDS, molecular biology grade, in ddH_2O.
7. 50 mM Tris-(2-carboxyethyl) phosphine (TCEP) in ddH_2O.
8. 200 mM Methyl methanetiosulfonate (MMTS), or 200 mM iodoacetamide, both in isopropanol.
9. 0.5 M Triethylammonium bicarbonate, pH 8.5 in ddH_2O.
10. 1 M KCl in ddH_2O.
11. Cation exchange loading buffer for chromatographic peptide clean up: 10 mM KH_2PO_4 in 25 % acetonitrile.
12. Cation exchange chromatography cartridge cleaning solution: 10 mM KH_2PO_4, pH 3.0 in 25 % acetonitrile/1 M KCl.
13. Chromatographic elution buffer: 10 mM KH_2PO_4, pH 3.0 using 10 mM K_2HPO_4 in 25 % acetonitrile/350 mM KCl.
14. Trypsin of the highest quality that is treated with l-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (*see* Section 3.3). We have successfully used both Applied Biosystems and Sigma-Aldrich. Also, *see* **Note 1** on optional use of immobilized trypsin systems. Whatever the source of trypsin, it must be mass spectrometry grade.
15. Acetone of high purity, store at room temperature.
16. Dissolution buffer: 0.5 M triethylammonium bicarbonate, pH 8.5.

3 Methods

Protocols such as those described herein are becoming more commonplace in modern neuroscience research, and many modifications exist when pursuing slightly different goals. Thus, what we describe here should be recognized as simply the beginnings of the dive into proteomic space. However, it deserves reiteration here that without proper handling of samples prior to generation of the spectra and quantification of the results, all downstream applications will be compromised. Thus, it is with this in mind that we attempt here to get the investigator off to a good start by informing them of this technology and the major potential pitfalls along the way.

Conceptually, the labeling of a sample for iTRAQ analysis is straightforward, and uses well characterized chemistries familiar to most protein chemists. As detailed in the stepwise workflow diagramed in Fig. 3 and explained in detail in Section 1.1, the methods describe the following steps:

- Generate protein lysates.
- Reduce disulfide bonds.

- Block the reactive cysteines.
- Digest the proteins to their constituent peptides.
- Differentially label the peptides of each sample with one of the iTRAQ reagents.
- Combine samples.
- Submit for mass spectrometry analysis and analyze results.

Sample preparation, processing, and labeling will be covered stepwise. However, only general points of mass spectrometry data analysis will be covered, due to the variations and complexities introduced by experiment specific issues. We suggest consulting mass spectrometry core personnel. The goal here is to alert and inform the reader to issues directly under the control of the biologist that can impact downstream analysis so that properly informed decisions can be made where necessary.

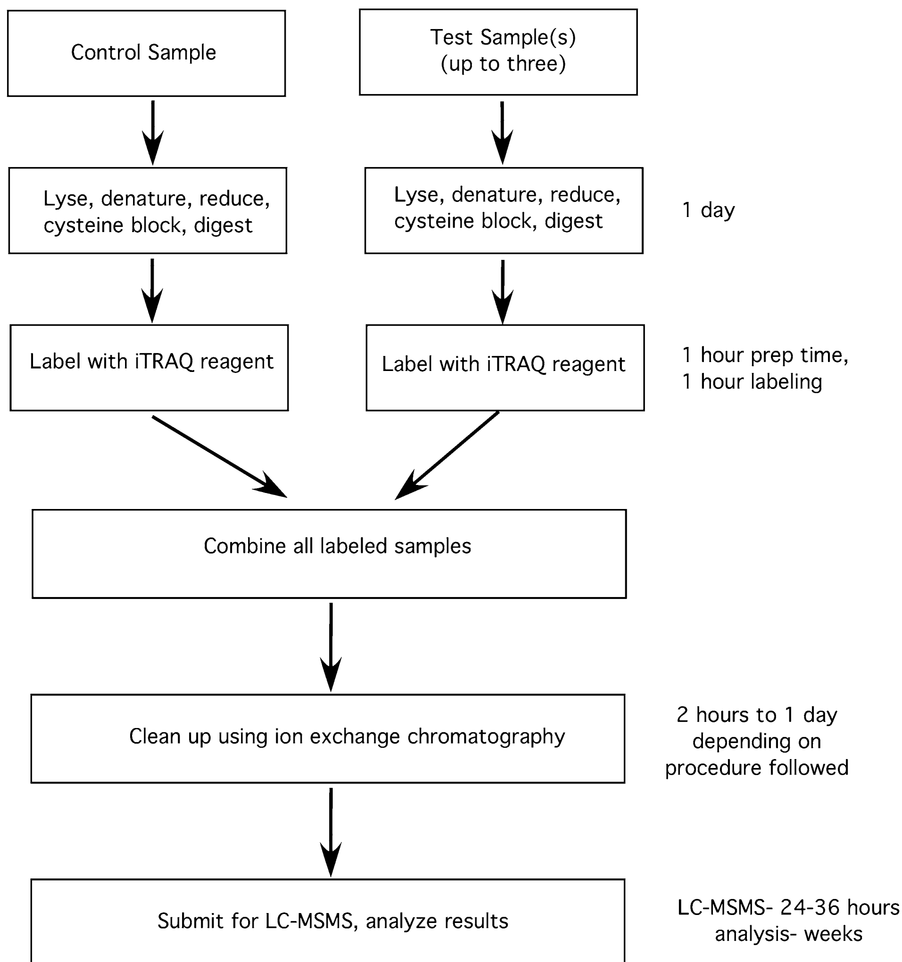

Fig. 3 Flow diagram for iTRAQ labeling procedure. See text for specific information on each step

3.1 Sample Preparation

3.1.1 Cell Culture

1. Generate samples by manipulating flasks/dishes of cells as required for the experiment (i.e. drug application, activation by bioactive molecules, etc.). Keep samples separate through these steps. We use OC-K3 cells, but the steps detailed here are useful for any cell line or primary cell culture. Numerous cell lines are now available for those interested in cell biological processes of auditory system-derived cells [24, 25].
2. Wash cells gently in ice-cold HBSS 2×.
3. Remove HBSS each time by aspiration, but do not allow the cells to dry. It is vitally important to carry out the lysis steps at 4 °C to inhibit proteolysis.
4. Immediately add the minimal volume of lysis buffer needed to cover the cells, and incubate on ice for approximately 10–20 min (*see* **Note 2**).
5. Scrape and transfer cell/lysis buffer solution to an appropriate sized tube. Usually a 1.5–2 mL snap top eppendorf tube will suffice, but this will depend on the size of the dish being lysed.
6. Optional step: Sonicate, on ice, to further disrupt cells, denature genomic DNA, and decrease viscosity of sample.
7. Centrifuge at 18–20,000 × *g* in a refrigerated centrifuge at 4 °C for 20 min to pellet cellular debris.
8. Transfer supernatant to fresh tube. Lysate should optimally be used immediately, but can be stored at −80 °C as required.
9. Estimate protein concentration (*see* **Note 3**). For future use, have on hand approximately 250 μg–1 mg of protein per sample condition. Adjust the concentration of the sample to 10 mg/mL. The final protein lysate can be stored at −80 °C for future use.

3.1.2 Tissues (*See* Note 4)

1. Process tissue to remove blood by transcardial perfusion of ice-cold saline.
2. Dissect quickly and immerse in lysis buffer at 4 °C in a tissue grinder.
3. Grind tissue to fully lyse the sample. When isolating cochlea, care should be taken to extract any cerebellar tissue from the recess holding the flocculus. Entire cochlear samples can be prepared, or cochlea microdissected without the bone capsule prior to lysis.
4. An optional step can be performed utilizing a probe sonicator. Sonication must be performed at 4 °C (*see* **Note 5**).
5. Follow Section 3.1.1, **steps 6–9**.

3.1.3 Phosphoprotein Enrichment

At times, the investigator may wish to assess post-translationally modified proteins. Numerous possibilities exist for enriching a sample for proteins/peptides that have undergone post-translational modifications. *See* **Note 6** for details.

3.2 Sample Processing (See Note 7)

3.2.1 Acetone Precipitation

Standard acetone precipitation techniques can be used to isolate the proteins of a sample from potentially interfering substances, or to concentrate the sample.

1. Transfer sample to a tube that can hold approximately 10× the sample volume.
2. Chill both the sample and the acetone to 4 °C.
3. Add six volumes of the cold acetone to the sample in the larger tube.
4. Cap the tube, and invert the sample 3–5× to thoroughly mix the sample and acetone.
5. Incubate the tube at −20 °C for 4 h. A precipitate should become clearly visible.
6. Briefly centrifuge at 4 °C to pellet the precipitate (18,000 × *g* for 2 min).
7. Decant off the acetone from the tube and proceed immediately to the reducing and blocking steps (Subheading 3.2.2). Do not allow the pellet to dry.

3.2.2 Denaturing, Reducing, and Cysteine Blocking the Sample (See Note 8)

1. Add 20 μL of dissolution buffer per tube containing up to 100 μg of protein sample or the acetone precipitated pellet. In order to have ample sample for labeling and processing, one may wish to label 250 μg of protein. Simply scale up the reaction as required (*see* **Note 9**). Add 1 μL of the denaturant (2 % SDS).
2. Check the pH of the solution by spotting some of the sample onto pH paper. The pH should be above 8.0.
3. Vortex to mix well, but avoid formation of bubbles/foam. Spin at low speed if necessary to eliminate foam.
4. Add 2 μL of reducing agent (50 mM TCEP) and vortex again to ensure complete mixing.
5. Incubate tubes at 60 °C in a heat block for 1 h.
6. Pulse spin briefly to collect sample at the bottom of the tube.
7. Add 1 μL cysteine blocking reagent (200 mM MMTS or iodoacetamide).
8. Vortex to mix, and incubate at room temperature for 10 min.

3.3 Trypsin Digest (See Note 10)

3.3.1 Digesting Samples with Trypsin

1. Dissolve trypsin at 1 μg/μL in ddH_2O or if using trypsin from the iTRAQ kit, use 25 μL Milli-Q standard H_2O per tube. If preparing more than two samples for analysis, prepare 50–60 μg of trypsin, or two tubes from kit.
2. Vortex to mix thoroughly.

3. To each 100 μg of sample in dissolution buffer from Subheading 3.2.2, **step 8**, add 10 μL of trypsin solution. Keep the final volume below 50 μL, or SpeedVac® as appropriate prior to the addition of trypsin.
4. Vortex to mix thoroughly.
5. Incubate at 37 °C for 16 h (overnight).

3.4 Labeling Samples with iTRAQ Reagents

The heart of the iTRAQ technology lies in the ability of the investigator to efficiently and completely label each peptide in a sample with a traceable, quantifiable tag. Each sample is processed separately to label the peptides.

1. Equilibrate the iTRAQ reagent to room temperature before use.
2. Add 70 μL of absolute ethanol to each iTRAQ label tube that will be used.
3. Vortex approximately 1 min to ensure complete mixing and dissolving.
4. Transfer the entire volume of one iTRAQ label reagent tube to one sample tube such that each sample tube receives one iTRAQ reagent. Usually, controls are labeled with the 114 reagent, but of course this is up to the investigator. It is critical to keep track of which sample received which label, as downstream quantification depends on the proper ratio of control to manipulated state.
5. Vortex to mix, and then pulse spin to collect all solutions to the bottom of the tube.
6. Incubate at room temperature for 1 h.
7. Combine the label reactions into one tube, such that a single tube receives each label singly, without duplication of labels if doing more than four labels (for the 4-plex kit, 8 if using the soon to be released 8-plex kit).

3.4.1 Assessing Completeness of Labeling Reaction (See Note 11*)*

1. Follow Subheading 3.4, **steps 1–6**, but only label one digested sample, using the 114 label.
2. Dry sample in a SpeedVac® and resuspend in 20 μL of 0.5 % trifluoroacetic acid (TFA) in 5 % acetonitrile per 30 μg total peptide.
3. Calculate the number of columns required. The number of columns needed depends on the amount of peptide being run. The binding capacity of each column is 30 μg, so calculate the number relative to the binding capacity of the columns and the amount of peptide used.
4. Wet the resin of the spin column with 200 μL of 50 % methanol.

5. Spin at 1500 × *g* for 1 min.
6. Repeat **steps 4** and **5**, discarding the flow-through each time.
7. Add 200 μL of 0.5 % TFA in 5 % acetonitrile to each spin column.
8. Spin at 1500 × *g* for 1 min and discard the flow-through.
9. Repeat **steps 7** and **8**.
10. Apply the labeled sample onto the bed of the spin column.
11. Spin at 1500 × *g* for 1 min at room temperature.
12. Set flow-through aside in case sample binding needs to be verified.
13. Apply 200 μL of 0.5 % TFA in 5 % acetonitrile to wash the column and spin at 1500 × *g* for 1 min.
14. Discard flow-through and repeat **step 13**.
15. Elute the sample from the column by adding 20 μL of 70 % acetonitrile to the bed of the column and centrifuging at 1500 × *g* for 1 min.
16. Repeat **step 15** with fresh 70 % acetonitrile.
17. Dry the sample in a SpeedVac®.
18. Resuspend the sample in 30 μL dissolution buffer.

Repeat the labeling reaction as outlined in Subheading 3.4, **steps 1–6**, using the 117 iTRAQ labeling reagent (*see* **Note 12**).

3.5 Preparing the Combined Sample for Mass Spectrometry (See Also Note 13)

The entire volume of all samples is combined in a 1:1:1:1 ratio. The most common method of analyzing the iTRAQ sample is via LC-MS/MS. Thus, the sample must be prepared so that it will be efficiently nebulized during the electrospray introduction into the mass spectrometer. At this stage, it is recommended that personnel who run the samples through the mass spectrometry be consulted, because the type of LC one performs will dictate to some degree the final preparation of the sample. A clean up procedure of the sample is described here as a general guide.

1. Assemble the cation exchange system as per manufacturer's instructions. Most important is the fitting between the syringe and the needle to port adapter. Be sure that the system is snug to keep solutions from backing up and out of the system.
2. Dilute the concentration of undesirable materials such as salts and detergents by adding ten volumes (relative to the sample) of loading buffer.
3. Vortex to mix thoroughly.
4. Spot an aliquot onto pH paper to ensure the pH of the solution. The pH must be between 2.5 and 3.3. Add more loading buffer as needed to attain the proper pH (*see* **Note 14**). Prepare the

column to accept the sample by injecting 1 mL of conditioning buffer. Allow flow-through to go to waste. All injections should be made with a slow and steady pace.

5. Inject 2 mL of loading buffer into the cartridge and divert flow-through to waste.
6. Inject diluted sample at a rate of approximately 1 drop/s into the column. Collect the flow-through and save until sure that the peptides were maintained on the column.
7. Inject 1 mL of loading buffer to wash the column. Save in the same tube as used in **step 7**. This step may be repeated as necessary.
8. Elute retained peptides by slowly (~1 drop/s) injecting 500 μL of elution buffer. Collect the eluate into one tube.

3.6 Mass Spectrometric Analysis of iTRAQ-Labeled Samples

The iTRAQ system makes use of isobaric tagging reagents (Fig. 2b) that carry a highly efficient and accurate peptide reactive group, a balance group, and one of a series of reporter groups of differential mass that is capable of maintaining its charge. The tags co-elute during typical reverse phase liquid chromatography performed prior to injection into the mass spectrometer, and are cleavable under MSMS conditions. Upon cleavage in the collision cell of a mass spectrometer, the tag is released from the peptide, thereby giving rise to two important regions of the spectrum: (1) the sequencing portion of the spectrum, where the data for peptide sequence are maintained; and (2) the reporter region of the spectrum, in which the isobaric tags are found. Figure 4 illustrates these regions as viewed in ProteinPilot (*see* **Note 15**). Finally, the reporter region is typically a relatively "quiet" region in the spectrum, and thus one can be confident that the peaks observed are derived from the tag itself. If other peaks are present in the reporter region, this may be an indication of noise resulting from poor sample processing, contaminants, etc.

3.7 What to Do with All of Those Data

Both proteomic and genomic analyses are wonderful in that one receives so much data. However, therein also lies the problem (and the danger)—how to verify the results, and what to do with the results once verified. Lists of proteins expressed are useful, but only at a superficial level. Verification can take the form of western blot analysis, follow-up proteomic analysis, etc. Bioinformatic approaches to proteome analyses are maturing at a steady and ever increasing pace. At this stage, of course, the investigator must decide in which direction to proceed. Listed in **Note 15** are URLs that can be used as a jumping off point to investigate various databases and software. Of particular interest are those that detail methods by which protein:protein interactions are modeled [26–29], and tools with which to generate and investigate interaction pathways [30–32].

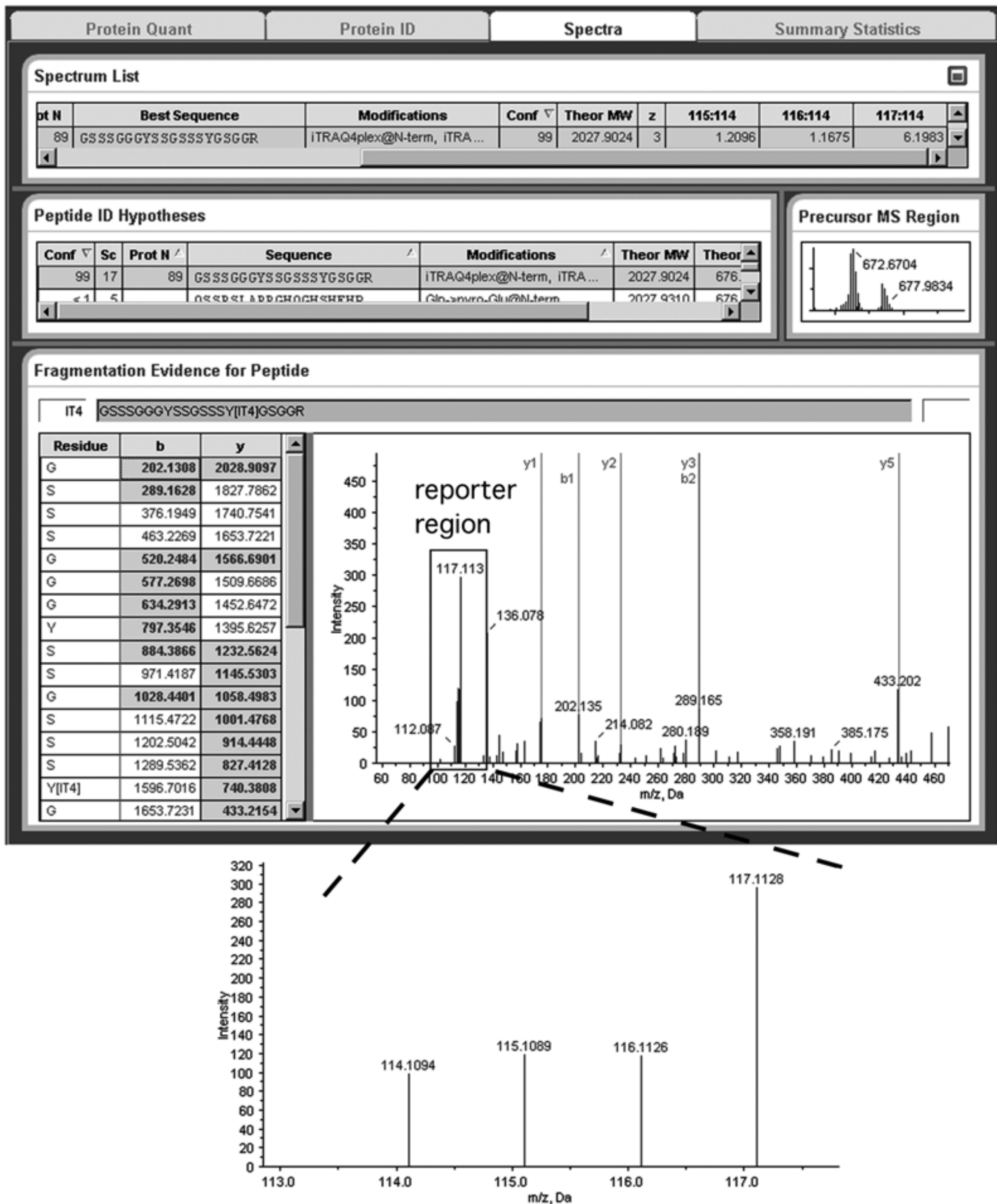

Fig. 4 ProteinPilot screen shot of typical results from an iTRAQ experiment. When examining the spectra of a peptide of interest (spectrum list), ratios of labels are indicated (here, 114 was used as the normal state, and therefore the denominator for the ratio). Additionally, the spectrum is inspected for manual annotation and analysis if desired in the fragmentation window. Regions can be zoomed in as indicated here to visually inspect the reporter region of the spectrum (*dashed lines*). In this case, this peptide was upregulated only during conditions in state 3 (~6× upregulated), while under conditions 1 and 2 (labeled with 115 and 116 reporters, respectively), no change is detected from baseline (label 114)

4 Notes

1. Immobilized trypsin can be obtained from Pierce or Sigma in which trypsin is cross-linked to agarose beads and is present as a slurry. To use immobilized trypsin, follow these steps:
   - Wash 100–250 μL of the trypsin bead slurry with 500 μL of water or a trypsin digestion buffer without primary amines. Repeat for a total of three times, each time centrifuging the beads down (~2000 × *g*) and decanting or pipetting off the supernatant. Be sure to thoroughly resuspend the beads with each wash and vortex to ensure complete mixing.
   - Resuspend the slurry in 200 μL of water or a trypsin digestion buffer without primary amines.
   - The sample should be resuspended in water or a trypsin digestion buffer without primary amines at a final concentration of 2 μg/μL.
   - Add the sample to the resuspended trypsin slurry.
   - Vortex to ensure complete mixing.
   - Incubate 16 h (overnight) at 37 °C with end-over-end mixing if possible.
   - Isolate the digest by spinning the slurry down and collecting the supernatant.
   - SpeedVac® the digest and bring back up in dissolution buffer to 30 μL.
   - Any formulation of lysis buffer should suffice (RIPA buffer, T-Per or M-Per from Pierce, etc.), and the exact composition of the lysis buffer will depend on the proteins targeted for analysis. For example, isolation and analysis of membrane proteins will demand a different lysis buffer from the standard lysis buffer for cytoplasmic proteins. Usually, the differences between lysis buffers are in detergent and/or osmolarity used. No protease inhibitors should be present, as this will inhibit the tryptic digest in downstream steps.
2. We have had good success with the Pierce MicroBCA kit.
3. Tissues represent a more difficult sample to deal with, owing primarily to the greater complexity of tissue compared to cells in culture. However, this should not dissuade one from such experiments, but rather alert one to the potential pitfalls associated with working with highly complex material. One major determinant of the success of proteomic analysis of tissue samples is the presence and successful depletion of plasma in the sample. Plasma is in such abundance that it often times masks proteins from detection. Various methods exist for depleting

tissue samples of plasma (or at least lessening the contribution of plasma to the protein mixture). For simplicity, we suggest first perfusing ice-cold saline transcardially through the animal in an attempt to clear the blood while also slowing proteolysis. Here, the investigator may need to adjust the procedure depending on the requirements for the experiment.

4. The advantage to sonication is the resultant decrease in viscosity due to the breakdown of the genomic DNA. Experience with the tissue of interest will reveal whether this is a significant problem to be addressed.
5. Many proteins are regulated by post-translational modifications. One of the most studied modifications is the addition of phosphate groups to distinct residues of proteins. If one is interested in assessing phosphorylation states on proteins in the samples, various enrichment protocols can be followed. The reader is directed to the Pierce Phosphoprotein Enrichment Kit (cat# 90003) as a potential starting point, although others exist from different vendors. These columns are simple to use, and yield a significant enrichment of phosphoproteins, but at the expense of losing non-phosphorylated proteins from the sample.
6. Numerous substances in the sample mixture can potentially interfere with the final labeling reaction. Chief among these are amine-containing compounds such as ammonium sulfate, -bicarbonate, -citrate, etc. These will interfere with the labeling process by competing for the iTRAQ label. Other issues to be concerned with are the presence of thiols that are typically introduced into sample mixtures by the addition of DTT or 2-β-mercaptoethanol (2βME), which can interfere with the cysteine blocking steps, and high amounts of detergents or denaturants, which will interfere with the tryptic digest step. Should any of these be present in the sample, one must perform a standard acetone precipitation step to isolate the sample from the interfering substance. If DTT or 2βME are present, the acetone precipitation should be performed immediately prior to moving on further in the procedure. If either detergents or primary amines are present, acetone precipitation can be performed either after reducing the sample and blocking the free cysteines (in the case of detergents that might be necessary for maintaining sample solubility), or just prior to tryptic digest (in the case of the presence of primary amines). The best practice is to avoid the need for precipitation at all, as this step represents a major point in the overall procedure where uncontrolled sample loss can occur, thereby, introducing bias in the relative amounts of proteins present in each sample. However, precipitation cannot always be avoided. If the trypsin digest will employ an immobilized trypsin (*see* **Note 1**),

acetone precipitation will be required, and the sample should be resuspended only in ddH_2O or a buffer without primary amines. In this case, the acetone precipitation should be performed after denaturing, reducing, and cysteine blocking steps detailed in Subheading 3.2.2. Prior to acetone precipitation, one may also wish to enrich for phosphoproteins (*see* **Note 6**). On occasion, the sample may be insoluble in the dissolution buffer concentration plus SDS as added. Should this be a problem, the dissolution buffer volume can be increased up to 50 μL without adversely affecting the labeling step. If more is required, one should first SpeedVac® the sample to near dryness, and add up to 100 μL of dissolution buffer. Should this still not solve a problem of an insoluble sample, two alternatives exist. One may either use a detergent/denaturant or a different buffer. An addition of 1 μL of 2 % SDS per 20 μL of the dissolution buffer will keep the concentration of SDS low enough so as to not interfere with the future trypsin digest. Use of denaturants or detergents other than SDS are possible, and included octyl β-D glucopyranoside (OG), NP-40, Tween-20, Triton X-100, and CHAPS (all at less than or equal to a 1 % final concentration), or urea at less than 1 M. In all cases, the aqueous partition of the sample must not be higher than 40 % to avoid problems with final labeling. Any other buffers tried should not carry primary amines, and should buffer at pH 8.0–8.5. These include, but are not limited to, BES, BICINE, CHES, HEPES, MOBS, MOPS, and PIPES. Concentration should be kept at approximately 0.3 M such that at the labeling step, the buffer concentration does not fall below 0.06 M.

7. In order to best label the proteins in the sample, one first must denature the sample to allow equal access to all possible modifiable amino acids carrying primary amines. Because denaturing with chemicals such as SDS will unfold proteins, but not destroy secondary structure induced by the presence of disulfide bonds, the denatured proteins must then be chemically reduced, generally using either DTT or some other reducing agent. The iTRAQ kit uses tris-(2-carboxyethyl)phosphine (TCEP) for this purpose. TCEP is used to denature the sample so that thiols are not introduced to the sample (*see* Subheading 3.2). Finally, the reduced cysteines must be blocked. Typically, this is done with iodoacetamide (at a concentration of 200 mM in isopropanol). One may use this, but the iTRAQ kit includes methylmethane-thiosulfonate (MMTS, 200 mM in isopropanol) for cysteine reduction. The advantage of MMTS is that it is a reversible blocker that can be useful should one decide to fractionate the sample by selectively isolating cysteine-containing peptides. We have found the procedure employed by the iTRAQ kit to be easy to follow and to yield excellent labeling.

8. It is critically important, however, to not allow the protein concentration to significantly decrease due to an increase in volume. If the sample is insoluble at a final concentration of 5 mg/mL, *see* **Note 7** for alternatives.
9. Trypsin is a pancreatic serine endoprotease derived from pancreatic trypsinogen following a removal of its N-terminal leader sequence. Trypsin is highly selective in its cleavage of peptide bonds, and cleaves only those bonds in which the carboxyl group is contributed by arginine or lysine. When trypsin is treated with l-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK-treated), any contaminating chymotrypsin activity is irreversibly inhibited. Additionally, when the lysine residues of trypsin are modified by a reductive methylation process, autolytic cleavage of trypsin is also irreversibly inhibited. This results in a loss of tryptic peptide fragments in the mass spectra. Neither treatment alters the trypsin activity toward the proteins present in the lysates. TPCK modification is especially important in iTRAQ-labeled mass spectrometry. Contaminating chymotryptic digests result in cleavage of peptides at the carboxyl side of tyrosine, tryptophan, and phenylalanine (i.e. amino acids containing phenyl rings). With longer digests, chymotrypsin will also hydrolyze other amide bonds, particularly those with leucine-donated carboxyls. These digests do not guarantee that a modifiable residue will be present in each resultant peptide. Following tryptic digestion, one can be sure that each peptide generated possesses at least a single modifiable amino acid that can be labeled by the iTRAQ reagents. Thus, TPCK-modified trypsin should always be used. Immobilized trypsin (*see* **Note 1**) is also especially useful, since autolysis, and therefore spectral peaks derived from trypsin, is virtually eliminated. However, immobilized trypsin is not provided with the iTRAQ kit. The investigator should determine the need for elimination of trypsin-derived peaks and balance the extra effort/cost with the desired results.
10. The first time one performs the iTRAQ labeling procedure, it may be worth the added effort to first assess one's success in fully labeling a sample. Without complete labeling, some peptides will be unlabeled and not represented in the reporter region of the spectra (see further discussion below) despite the fact that the peptides are actually present in the sample. A simple test can be performed to assess the completeness with which the sample is labeled with the aid of C18 spin columns such as that from Pierce (PepClean C18 columns). These columns are good general tools for purifying and concentrating peptide samples.
11. If the initial labeling reaction was not carried to completeness, the sample should contain both the 114 and the 117 labels in the mass spectrum analysis. As long as no 117 label is detected,

the initial labeling reaction can be considered complete, and there would be little need to further analyze labeling efficiency in the future unless significant changes to sample preparation are encountered.

12. Many compounds potentially present in the sample can interfere with the LC-MS/MS analysis. These include, but are not limited to, the dissolution buffer, ethanol, TCEP, any salts, excess iTRAQ labeling reagent, denaturants, and detergents. Thus, a simple method for cleaning the sample needs to be used. Cation exchange chromatography can be used to isolate the sample from interfering compounds and allows one to replace the sample diluent with a more "mass spec friendly" diluent. A variety of cation exchange systems can be purchased. One system that uses spin column and vacuum plate technology for ion exchange chromatography is the VivaPure system (Sartorius Biotech, Inc., Goettingen, Germany). The system appears straightforward to use, but we have had no first-hand experience with this. Included in the Applied Biosystems Methods Development Kit is a system for performing cation exchange chromatography on the sample prior to submission for mass spectrometry. However, this system is useful only for relatively simple samples such as that obtained from phosphopeptide-enriched samples, samples that have undergone some other type of affinity enrichment, or samples from cell culture. Complex samples such as those obtained from tissue lysates will require more complicated fractionation, perhaps even over a two-dimensional LC system, prior to MS analysis. For complex samples, or if more complex fractionation is desired (thus allowing one to uncover signatures of the less abundant proteins in the sample), the appropriate personnel should be consulted.

13. The cation exchange column will only work properly when the peptides are carrying a cationic charge. The charge allows them to adhere to the column matrix while inorganics, such as salts, and organics such as acetonitrile, can be flushed away.

14. A viewer version of Protein Pilot can be obtained as freeware distributed by Applied Biosystems for use with data generated by their QStar and QTrap mass spectrometers. The exact file type the investigator will receive back from the mass spectrometry facility will depend on the type of instrument used, local preferences for software, etc. Here, we will only present analysis performed with the Protein Pilot software. However, other software will perform in a similar manner. Data represented in ProteinPilot include proteins detected in the sample, the ratio of tags for that protein, individual peptide sequence and tag ratios, and a spectral characterization of tag intensities. From these four simple windows, one can quickly assess relative protein concentrations between the samples analyzed.

However, one should also inspect the protein identification window to make investigator-based decisions concerning identifications. In the best cases, one should expect detection of multiple peptides of the protein being identified and these peptides should span numerous regions of the protein. Thus, peptides from N- and C-term regions, as well as internal regions should be present to allow for the best identification. However, this is not always available, and so further investigation in these cases needs to be done to assess the veracity of the identification in light of incomplete coverage.

15. Useful URLs detailing protein interaction databases, and pathway visualization and structure analysis include:
    - Human–protein interaction database [33], http://www.hpid.org;
    - Human–protein reference database, http://www.hprd.org;
    - Mammalian protein–protein interaction database (MIPS);
    - http://mips.gsf.de/proj/ppi;
    - Molecular interactions database (MINT) [34], http://mint.bio.uniroma2.it/mint;
    - Proteomics Identifications database (PRIDE) [35, 36], http://www.ebi.ac.uk/pride;
    - Protein–protein interaction network visualization software Cytoscape [37] GenePro plug-in for Cytoscape, http://genepro.ccb.sickkids.ca/index.html;
    - ProViz http://cbi.labri.fr/eng/proviz.htm.

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