**TECHNICAL BRIEF**

**A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen**

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In order to assess the biological function of proteins and their modifications for understanding signaling mechanisms within cells as well as specific biomarkers to disease, it is important that quantitative information be obtained under different experimental conditions. Stable isotope labeling is a powerful method for accurately determining changes in the levels of proteins and PTMs; however, isotope labeling experiments suffer from limited dynamic range resulting in signal change ratios of less than ~20:1 using most commercial mass spectrometers. Label-free approaches to relative quantification in proteomics such as spectral counting have gained popularity since no additional chemistries are needed. Here, we show a label-free method for relative quantification based on the TIC from peptide MS/MS spectra collected from data-dependent runs can be used effectively as a quantitative measure and expands the dynamic range over isotope labeling experiments allowing for abundance differences up to ~60:1 in a screen for proteins that bind to phosphotyrosine residues.

**Keywords:**
Differential expression / Quantitative analysis / Shotgun proteomics / Signal transduction profiling

Methods for acquiring quantitative proteomics data are continually developing with very accurate stable isotope labeling (SIL) and label-free approaches. SIL provides chemically equivalent but isotopically different internal standards for each peptide/protein for direct comparison of mass spectral signal intensities that represent relative abundance.

Common SIL strategies include protein level labeling strategies such as stable isotope labeling of amino acids in cell culture (SILAC) [1], a global method whereby all translated proteins have isotope labels metabolically incorporated at selected amino acid residues, and isotope-coded affinity tags (ICAT) [2], a technique that labels cysteine residues at the protein level. Peptide level labeling strategies include multiplexed isobaric tags for relative and absolute quantification (iTRAQ) [3], global internal standard technology (GIST) [4], a global post-digestion labeling method that labels primary amine groups (peptide N-terminus and lysine residues) and an extension of GIST called in-gel stable isotope labeling (ISIL) [5, 6], a method that labels primary amine groups of proteins (protein N-terminus and lysine residues) directly from gel separated samples. Each isotope labeling strategy has its advantages and disadvantages depending upon the experimental questions but most suffer from an experimental...
dynamic range limitation of ~20:1. Protein level differences greater than this limit usually suffer from very large errors in ratio determination [7].

Label-free approaches to quantitative proteomics have gained prominence in recent years since no additional chemistry or sample preparation steps are required. These include biostatistical profiling [8, 9] in clinical proteomics whereby peptide ions that show intensity changes over many samples are recorded and represent potential protein biomarkers, methods for comparing the ratios of integrated peak areas/intensities of phosphorylated to nonphosphorylated peptide ions between two samples [10, 11], a strategy to assess the stoichiometry of phosphorylation sites [12], and the popular method of quantifying protein levels from different samples based on the number of MS/MS spectra that identify the protein of interest (spectral counting) [13–17].

We demonstrate a method to survey relative protein quantity between samples that is an extension to the spectral counting technique whereby the average of the TIC for all of the MS/MS spectra that identify a protein is used as a quantitative measure. Each spectral count gets a unique abundance value rather than equivalent values of “1” as with spectral counting. This “spectral TIC” method is unique and expands the dynamic range of quantitative ratios allowing for larger protein abundance differences than the ~20:1 practical limit obtained using SIL since some biological systems demonstrate very large protein expression differences. For example, the proto-oncogene products c-jun and c-fos show expression level increases of 38 and 72-fold, respectively, under oxidative stress conditions [18]. Additionally, several other proteins in biological systems such as glucokinase in metabolism demonstrate very large fold changes in protein expression levels [19, 20].

Spectral TIC method: protein pools from at least two different experimental conditions were digested with trypsin and the peptide mixtures were microcapillary LC-MS/MS using an LTQ 2-D linear IT (ThermoScientific, San Jose, CA) operated at a flow rate of 300 nL/min in positive ion dependent acquisition (DDA) mode with a repeating cycle of one survey MS scan followed by eight MS/MS scans with a 2 min exclusion window collected over a 50 min gradient (4–38% B: ACN, A: 0.1% formic acid/99.9% water). The TIC data from each MS/MS spectrum were obtained from the raw datafile and relative quantitative ratios were calculated by taking the average TIC from all of the MS/MS spectra identifying each protein including all redundancy, charge states, and missed cleavages, and then dividing the experimental sample (numerator) by the control sample (denominator). The TIC from the reverse database were accepted if at least two unique peptides were identified from a SEQUEST search against the reversed NCBInr protein database with a PBS consensus score greater than 1.00.

To show that spectral TIC compares well to spectral counting and is capable of increased dynamic range, two different concentrations of a BSA digestion (50 fmol and 1 pmol) were injected onto the LC-MS/MS system and SEQUEST identified 54 and 206 trypptic peptide spectra, respectively. These numbers represent the spectral counts. The spectral counting ratio was 3.8:1 though a 20:1 concentration difference was experimentally created. If one averages the TIC values for all of the BSA tryptic peptide spectra from each experiment, 7.9E5 (1 pmol digest) and 3.8E4 (50 fmol digest), the ratio is 20.8:1, a value very close to the actual 20:1 abundance ratio while the spectral counting method did not produce an accurate ratio. To support the result, the area of the reconstructed ion chromatograms for several peptides from each experiment from the MS scan was calculated and the average ratio was 17.9:1 for the 1 pmol:50 fmol (20:1) digestion mixtures supporting the accuracy of the method. One of the disadvantages of spectral counting is that the length of the protein influences the number of theoretical peptides that can be produced from tryptic digestions. The difference in quantitative accuracy between spectral counting and spectral TIC for the BSA example was due to the fact that at 50 fmol of digest, the theoretical limit of the number of possible detected peptides was approached with 62.1% amino acid coverage compared to 78.9% amino acid coverage for the 1 pmol digest given the high sensitivity of the LTQ IT. In these cases, relative quantity can only be assessed by using peak intensity values in either the MS or MS/MS scans. We also tested the variability of the spectral TIC ratios by injecting 300 fmol of a BSA digest 14 consecutive times and calculating 7 ratios that should theoretically be 1:1. The ratios ranged from 0.8 to 1.4 with the average ratio of 1.1, a SD of 0.226, and a CV of 20.7%. While the accuracy may be less than SIL experiments, the ease of use, lack of additional chemistries, and increased dynamic range are great benefits.

Screen for phosphotyrosine (pTyr) binding proteins: to demonstrate the effectiveness of the spectral TIC approach for quantifying a wide range of protein abundance differences in real biological systems, a screen for proteins that bind to pTyr residues was performed. Hela cell lysates were run over two types of columns. One column contained a fixed pTyr residue at the center of a partially degenerate bioinactivated 13-mer peptide library bound to streptavidin beads. The control column was identical except for a fixed nonphosphorylated Tyr residue in the center position of the peptide library in order to assess protein binding differences based solely on the phosphorylated Tyr residue. Proteins were eluted from each column with 20 mM sodium phenyl phosphate, digested with trypsin and then separated into six fractions by strong cation exchange (SCX) chromatography using a Paradigm 2D-LC (Michrom Bioresources, Auburn,
CA) using 20–450 mM ammonium formate in 25% ACN over 30 min. Peptide mixtures were run by LC-MS/MS using the LTQ IT in positive ion DDA mode. Relative quantitative ratios were calculated using two methods. First, ratios of proteins from each column were calculated by using spectral counting. The degree of protein binding to the pTyr column was calculated by a ratio of the number of spectral counts from the pTyr column experiment divided by the number of spectral counts from the Tyr column experiment. Secondly, spectral TIC was used to calculate the relative quantitative ratios. Figure 1 shows the experimental workflow.

From the label-free pTyr screen, we show nine proteins including eight known src homology 2 domain (SH2) domain-containing proteins [21] and pyruvate kinase M2 (PKM2), a glycolytic protein that is critical for proliferating cancer cells [22]. To demonstrate an example of a specific pTyr binding protein acquired by the spectral TIC method, the 85kDa regulatory beta subunit of phosphoinositide 3-kinase (p85) resulted in a spectral counting ratio of 15.0:1 (15 spectra from pTyr column and 1 spectrum from the Tyr column) while the spectral TIC ratio was 16:1 from the average TIC values 1.60E from the pTyr column and 1.0E 4 from the Tyr column. For p85, the spectral counting and spectral TIC ratios were nearly identical. However, PKM2 showed a spectral TIC ratio of 59.3:1, a ratio nearly twice the spectral counting ratio of 31.5:1. As quantitative controls for the experiment, ratios were also calculated for many proteins that did not change in protein abundance between the two experimental conditions (no preference for pTyr) such as filamin, plectin, myosin 9, HSP-70, HSP-90 and many others. The spectral TIC ratios for these proteins were all approximately 1:1, suggesting nonspecific binding. The difference in the ratios is likely due to a dynamic range limit of the spectral counting method similar to the situation for the BSA digest example above.

Comparison to SILAC: spectral counting and spectral TIC were then compared with SILAC using the same experimental conditions whereby two pools of HeLa cells were grown with either light ($^{12}$C$_6$) or heavy ($^{13}$C$_6$) lysine and

Figure 1. The experimental workflow for collecting label-free spectral TIC relative quantitative data in a screen for pTyr binding proteins. In general, protein pools from different sources are digested separately, peptides separated by SCX, and peptide fractions analyzed by data dependent LC-MS/MS. The TIC values from all tandem mass spectra identifying a protein from each experimental condition are averaged and the spectral TIC ratio is calculated for relative quantification.
arginine amino acids. Heavy cell lysate was passed over pTyr column while light lysate was passed over the Tyr column. Bound proteins were eluted with phosphate buffer, combined and separated by SDS-PAGE. The entire lane was excised into ten gel pieces, digested separately with trypsin, and then run by microcapillary LC-MS/MS in DDA positive ion mode for both identification and quantification using a high resolution QSTAR Pulsar i hybrid qTOF mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA) with one MS survey scan followed by three MS/MS scans. For SILAC, proteins were identified from the MS/MS scans and average peak pair ratios were calculated from the MS scan using the Paragon algorithm in ProteinPilot software (Applied Biosystems). Many peptide sequences per protein (at least six) contributed to the average SILAC ratios. The nonspecific binding proteins showed ratios of ~1:1, very similar to spectral TIC ratios. A pTyr binding protein was determined by a heavy (pTyr column): light (Tyr column) ratio of at least 3:1, the same values used for spectral TIC. Using p85 as an example, an average heavy/light ratio of 11.1:1 was determined and the peptide ion pairs appeared as heavy singlet peaks since only the heavy form bound to the pTyr column while no or little p85 bound to the Tyr column. To demonstrate an extreme dynamic range limitation of SIL, an average SILAC ratio of 15:1 was calculated for PKM2 compared to the ratios of 59.3:1 for spectral TIC and 31.5:1 for spectral counting. Figure 2 shows a ratio plot for nine pTyr binding proteins as well as several control proteins for the three different methods of relative quantification (spectral counting, spectral TIC, and SILAC). The spectral TIC ratio is valid based on several methods including SDS-PAGE and Western blots [22]; however the SILAC ratio appears erroneously low due to the dynamic range limitation for SIL experiments with TOF based mass spectrometers. Spectral counting shows improved dynamic range over SIL, however, to a lesser extent than spectral TIC. Table 1 lists the parameters and calculated values used for the three quantification strategies.

The differences between the ratios of the pTyr binding proteins obtained from the screen can be due to several factors including cellular protein abundance and binding efficiency to the pTyr peptide library, and neither of these can be differentiated in these experiments. The binding of SH2 domains to pTyr is influenced by the amino acid sequence N-terminal and C-terminal of the pTyr residue [21]. Also, MS/MS TIC values from shotgun DDA experiments can be subjective based on several factors during the analysis, including previous peptide ions that were attacked for MS/MS in data dependent cycles, the chromatographic elution point when a peptide ion is selected for MS/MS and differences in ionization efficiencies. However, we have determined that if sufficient peptide MS/MS spectra per protein (≥5) are identified, the average TIC value from DDA MS/MS spectra is a valid indicator of a protein’s relative abundance. Since the average TIC values are calculated over many peptides per protein, the method can also be used to assess relative stoichiometry of protein complexes within a single sample. We have tested...
relative concentrations of tryptic digests of BSA and Annexin V mixed at 3:1 (600 fmol:200 fmol), 1:1 (300 fmol:300 fmol), and 1:3 (200 fmol:600 fmol) ratios and were able to determine the relative stoichiometric ratios using spectral TIC with a 19.7% CV for five LC-MS/MS injections of each mixture.

The isotope-free spectral TIC method demonstrates a simple method for assessing relative quantities of proteins in different samples without the need for additional chemistries or complex calculations. The data can be extracted from typical LC-MS/MS experiments. It is not limited in the number of samples that can be quantified per experiment as with many SIL approaches and the expanded dynamic range provides insight into the relative binding efficiency and cellular protein abundance from regulatory processes that show very large fold changes and biomarker discovery where proteins become highly elevated in the diseased state. While isotope labeling methods have great utility due to their accuracy of quantification, they have limited dynamic range so that very large differences in peptide/protein abundance cannot be assessed.

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