



Published in final edited form as:

J Proteome Res. 2013 April 5; 12(4): 1996–2004. doi:10.1021/pr400100k.

Improved Detection of Quantitative Differences Using a Combination of Spectral Counting and MS/MS Total Ion Current

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Abstract

Label-free quantitative strategies are commonly used in shot-gun proteomics to detect differences in protein abundance between biological sample groups. Here, we have employed a combination of two such approaches, spectral counting (SpC) and average MS/MS total ion current (MS² TIC), for the analysis of rat kidney mitochondria in response to metabolic acidosis. In total, forty nine proteins were observed to be significantly altered in response to metabolic acidosis (p-value < 0.05). Of these, 32 proteins were uniquely observed as significantly different by SpC, 14 by MS² TIC, and only 3 by both approaches. Western blot analysis was performed on a subset of these proteins to validate the observed abundance differences. This study illustrates the utility and ease of combining these two label-free quantitative approaches to increase the number of detected protein abundance differences in the shot-gun analysis of complex biological samples.

Keywords

Spectral counting; total ion current; label-free; quantitative proteomics; metabolic acidosis; MS/MS TIC; spectral TIC

INTRODUCTION

Mass spectrometry based quantitative profiling enable the observation of protein abundance differences between disease states and have been extensively utilized for the discovery of potential protein biomarkers.^{1, 2, 3, 4} The ability to detect quantitative differences within complex biological systems is essential to further our understanding of cellular processes and disease progression.⁵ The analytical approaches employed for relative protein quantitation can be grouped into two major categories, labeling and label-free.⁶ Examples of labeling approaches include isotope coded affinity tags (ICAT)⁷, stable isotope labeling by amino acids in cell culture (SILAC)⁸, tandem mass tags (TMT)⁹ and isobaric tag for relative and absolute quantitation (iTRAQ).¹⁰ While labeling methods provide accurate quantitation, they suffer from dynamic range limitations (Asara 2008). Furthermore, labeling methods require complex sample preparation, are limited in sampling size, have issues with

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Author Contributions

The manuscript was written through contributions of all authors.

Supporting Information

Supplemental figure 1

Supplemental figure 2

Supplemental figure 3

Supplemental figure 4

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incomplete labeling, are expensive, and involve time consuming data analysis.¹¹ Moreover, cell culture specific methods, such as SILAC, cannot be employed for the analysis of primary tissues which are often important for biomarker investigation in clinical studies.¹²

Label-free approaches have become widely employed for the detection of relative protein abundance differences due to their ease of use and diverse applications.¹³ They are ideally suited for large scale discovery experiments with the goal of detecting significant differences between biological states to drive follow up studies. Additionally, label-free methods have been shown to enable relative quantitation over a larger dynamic range as compared to stable isotope labeling approaches.¹³ One frequently employed label-free approach is spectral counting (SpC) in which relative protein abundance is measured by the number of MS/MS spectra identified for all the peptides assigned to a protein. Liu, et al. demonstrated that this spectral count value strongly correlates with protein abundance in a complex matrix.¹⁴ Additionally, SpC analysis has been shown to be highly sensitive for the detection of subtle changes in protein abundance.¹⁵ However, Old, et al. illustrated a major limitation of SpC analysis for low abundance proteins (< 4 spectral counts) where quantitative values were found to be inaccurate.¹⁵ While SpC can suffer from inaccuracy in the quantitative measurement (i.e. fold change) it is extremely reproducible and easy to implement.

Another example of label-free quantitation is the use of ion intensity as a quantitative measure. Most approaches base the quantitative value on the intensity of the parent ion peak which requires high resolution measurements to ensure neighboring peaks are distinguished.¹⁶ This strategy is highly accurate but requires high resolution precursor ion measurements. An alternative strategy is instead based on the average or sum of the total ion current of the MS/MS spectra (MS^2 TIC) assigned to all peptides for a given protein. This strategy is particularly useful for low resolution experiments (e.g. ion trap instrumentation). Fragment ion intensity measurements have been used in targeted quantitative approaches such as multiple reaction monitoring¹⁷ and non-targeted labeling approaches such as iTRAQ.¹⁰ Recently, two studies have successfully used either sum or average MS^2 TIC measurements in shot-gun proteomics experiments to identify phosphotyrosine binding proteins as well as to explore protein differences between total lung homogenates and lung endothelial plasma membrane subfractions.^{18,19} Asara, et al. demonstrated that MS^2 TIC displayed an improved dynamic range for detecting relative protein abundance changes as compared to SILAC.¹⁹ MS^2 TIC methods have also been shown to provide improved sensitivity (as compared with SpC) for the detection of relative changes in low abundance proteins.²⁰ However, MS^2 TIC is not as reproducible as SpC due to inherent variation in sampling across a chromatographic peak over all samples.

One of the primary advantages of a label-free approach is that the data required for the analysis is collected as part of the standard analytical workflow and further experimentation or sample preparation is not required. Thus, both SpC and MS^2 TIC analysis can easily be performed within the same experiment. Here, we present a detailed comparison of these two label-free approaches applied to the same dataset. Our results indicate that they are complementary and the use of both approaches increases the value of the experiment by detecting a larger number of proteins found to be significantly changing between biological states. This dual strategy is particularly useful for laboratories working with older or low resolution instrumentation which often do not yield a large number of biologically important candidates to explore in follow up studies.

The analysis presented here was performed in the context of a larger study focused on understanding proteome remodeling in the mitochondria of the proximal convoluted tubule during the renal response to metabolic acidosis.²¹ Briefly, metabolic acidosis is a common

clinical condition that is caused by a decrease in blood pH and bicarbonate concentration.²² Previous studies have established that expression of a few mitochondrial proteins, including key enzymes of glutamine metabolism such as kidney-type glutaminase (KGA)^{23, 24} and glutamate dehydrogenase (GDH)²⁵, are increased during metabolic acidosis.²⁶ Thus, the relative abundance changes observed for KGA and GDH were used as positive controls for this study. The results describe the comparison of mitochondrial proteins that exhibit significant differences in protein abundance between control and acidotic rat kidneys as determined by SpC and MS² TIC analyses.

MATERIALS AND METHODS

Proteomic sample preparation

The study compared mitochondrial fractions of proximal convoluted tubules isolated from kidneys of control rats (n=3) and 7 d acidotic rats (n=3). Control rats drank tap water and rats that were made acidotic for 7 d were provided with 0.28 M NH₄Cl as their sole source of drinking water. Proximal convoluted tubule isolation and mitochondria enrichment procedures were described in detail.²¹ All of the procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University. Both Bradford²⁷ and bicinchoninic acid (Pierce) protein assays²⁸ were performed to determine protein concentrations. Briefly, 50 µg of the mitochondrial proteins underwent acetone precipitation, were resuspended in 8 M urea and 0.2% ProteaseMAX surfactant (Promega), reduced with dithiothreitol, and alkylated with idoacetamide. The proteins underwent tryptic digestion for 3 h at 37°C and then stopped with 0.5% trifluoroacetic acid. Peptides were dried in a speed-vac and purified using a reverse phase C18 TopTip (Glygen). Purified peptides were dried and reconstituted in 50 µl of 0.1% formic acid/3% acetonitrile.

Mass spectrometry

Briefly, 10 µg aliquot of each sample was injected onto a strong cation exchange (SCX) column (ZORBAX BioSCX Series II, 300 µm × 35 mm, 3.5 µm column, Agilent) and peptides were eluted using 20 µl salt bumps of 10, 15, 25, 35, 50, 75, 150, and 500 mM NaCl. The peptides were further fractionated on a reverse phase column (1200 nanoHPLC, Zorbax C18, 5µm, 75 µm ID x 150mm column, Agilent) using a 90 min linear gradient from 25%–55% buffer B (buffer B = 90% acetonitrile and 0.1% formic acid) at a flow rate of 300 nl/min. Peptides were eluted directly into the mass spectrometer (LTQ linear ion trap, Thermo Scientific) and spectra were collected over a m/z range of 200–2000 Da using a dynamic exclusion limit of 3 MS/MS spectra of a given peptide mass for 30 sec and an exclusion duration of 90 sec. A window of 2.0 m/z was used for precursor ion selection and relative collision energy of 35 V for fragmentation. The compound lists from the resulting spectra were produced using Xcalibur 2.2 software (Thermo Scientific) with an intensity threshold of 5,000 and 1 scan/group. Duplicate 2D-LC analysis was performed for each biological sample and sample order was randomized before analysis. The addition of 2D-LC to a relative quantitation workflow has been shown to not effect quantitation and results in an overall increase in peptide identifications.²⁹

Bioinformatics

The tandem mass spectra (MS/MS) were searched against the Uniprot-KB *Rattus norvegicus* protein sequence database (74,140 sequence entries) which was concatenated with a reverse database using both the Mascot (version 2.3.02, Matrix Science) and SEQUEST (version v. 27, rev. 11, Sorcerer, Sage-N Research) database search engines. The following search parameters were used: average mass, peptide mass tolerance of 2.5 Da, fragment ion mass tolerance of 1.0 Da, complete tryptic digestion allowing two missed cleavages, variable modifications of methionine oxidation and lysine acetylation, and a fixed modification of

cysteine carbamidomethylation. Peptide identifications from both search engine results were combined in Scaffold 3 (Version 3.6.0, Proteome Software) using protein identification algorithms. Search results from all raw files associated with a biological sample (i.e. all 2D-LC fractions and duplicate injections) were summed within the Scaffold 3 software. Peptide probability thresholds of 90% and protein of 99% were applied with Peptide and Protein Prophet algorithms in Scaffold 3.³⁰ Proteins containing shared peptides were grouped by Scaffold 3, satisfying the laws of parsimony. Using a target decoy approach a peptide false discovery rate (FDR) of 0.2% was determined.³¹ Proteins that were identified by a minimum of 2 unique peptides in at least 1 biological replicate were considered confidently identified. Using these criteria, 901 total proteins were identified in all the samples.²¹

Label-free protein quantitation

Box plots were generated to assess data quality between technical and biological replicates. Variance in the biological replicates was assessed by determining the number of proteins identified per sample, the total spectra identified, and the total peptides identified (Figure S1). Correlation of biological and technical replicates was tested using Pearson's correlation test (Figure S2). The statistical analysis was performed using the R v2.15 statistics package (<http://r-project.org>) and DanteR v1.0.2 (<http://omics.pnl.gov/software/DanteR.php>).

MS/MS data to be used for quantitation was normalized between samples in Scaffold 3 by using the sum of the unweighted spectral counts for each sample to determine a sample specific scaling factor that was then applied to all proteins in that sample. The impact of this normalization on both SpC and MS² TIC values is illustrated in Figure S3. Quantitative analysis using SpC and MS² TIC were separately performed on the normalized data. The average MS/MS total ion current value was chosen because it has been shown to be less biased by protein length than the sum.¹⁹ For SpC analysis, a pseudo value of 1 was added to all normalized values to eliminate zero values. For MS² TIC a pseudo count of 2,753.8 was added to all normalized values. This number represents the lowest observed MS² TIC value in the dataset. To test for significant changes in protein abundance between treatments, the Student's t-test was performed on the normalized spectral count or MS² TIC values. For a protein to be included in the quantitative analysis the following requirements had to be met; there must have been at least two unique peptides in at least two out of three biological replicates and there must have been a sum of 10 spectra overall in the biological replicates. Manual validation of MS/MS spectra was performed for protein identifications that met these thresholds and showed significant ($p < 0.05$) change in abundance between treatments, but only had two unique peptide assignments. Criteria for manual spectral validation included the following: 1) a minimum of at least 3 theoretical y or b ions in consecutive order with intensities greater than 5% of the maximum intensity; 2) an absence of prominent unassigned peaks greater than 5% of the maximum intensity; and 3) indicative residue specific fragmentation, such as intense ions N-terminal to proline and immediately C-terminal to aspartate and glutamate. These conservative thresholds coupled with manual spectral validation ensure that the MS² TIC values are not largely influenced by signal from chimeric peptides. Fold changes were calculated as the ratio of the mean normalized SpC or MS² TIC for the three biological replicates for each condition. Data quality was also assessed using the Power Law Global Error Model (PLGEM) as implemented within the Scaffold software (Figure S4)³²

Western blot analyses

Additional mitochondrial fractions isolated from renal proximal convoluted tubules were prepared to enable validation of the results observed by SpC and MS² TIC analysis. For western blotting, control and acidotic samples containing 10 μ g of protein were separated by 8 or 10% SDS-PAGE. Western blot analysis was carried out as described previously.²¹

Additionally, the rabbit polyclonal heat shock cognate 71 kDa protein (HSP7C) (Abcam) antibody was used to validate the change identified by MS² TIC.

RESULTS AND DISCUSSION

The experimental workflow beginning with mitochondrial isolation from three control and three acidotic rat kidneys is illustrated in Figure 1. Key to this workflow is the use of two label-free relative quantitative approaches (SpC & MS² TIC) from the same set of mass spectrometry data. Spectral counts, by definition, are assigned in whole number increments where the total spectral count value for a protein is the sum (total number) of all spectra assigned to peptides from that protein. Conversely, MS² TIC values are by definition much larger values where the summation of the total ion current for the MS/MS spectra are averaged for the assigned peptides from a given protein. The use of this total ion current value for each tandem mass spectra allows for a more weighted value to be assigned to each spectrum. Both SpC & MS² TIC values can be easily extracted from any dataset without special or additional data acquisition.

Careful design of a label-free quantitative experiment is critical to accurately identify protein changes.^{33, 34} One metric of quantitative data quality is that the number of proteins, peptides, and spectra identified in each sample are similar. Variance in the raw data was assessed by calculating the coefficient of variance between samples (CV). The following % CV were calculated for number of proteins identified per sample (% CV for control 4.7 and acidotic 1.4), the total spectra identified (% CV for control 15.7 and acidotic 2.5), and the total peptides identified (% CV for control 20.4 and acidotic 4.1) (Figure S1). The number of identified proteins, peptides, and spectra were within 20% for all the samples, both control and acidotic. Pearson's correlation calculations reported R² values ranging from 0.89–1.00 between all the samples (Figure S2). Box plots for both pre and post-normalization of quantitative values from SpC and MS² TIC showed minimal variation and the effects of normalization (Figure S3). Overall, the data showed acceptable qualitative features for quantitative analysis.

Normalization was performed on both the SpC and MS² TIC values to account for analytical variations between samples. The Student's t-test was used to determine statistically significant changes in protein abundance (p-values < 0.05). Each protein with a significant p-value had to meet the additional requirement of a sum of > 10 spectra over the three biological replicates and have a minimum of two manually validated unique peptides in at least two biological replicates. Biological relevance is often assessed by calculating the fold change, although it relies solely on the ratio of the quantitative values, not the variance between samples. Therefore, fold changes were only calculated for those proteins meeting the above requirements and a fold change cut off was not applied.

Of the 901 proteins identified a total of 49 proteins were observed to be significantly altered (p-value < 0.05) in response to metabolic acidosis. Of these, 32 proteins were observed only in the SpC analysis (Table 1), 14 were observed only in the MS² TIC analysis (Table 2), and 3 were observed by both approaches (Fig. 2). The three common proteins, apolipoprotein A-IV, peroxisomal bifunctional enzyme (EHHADH), and glutamate dehydrogenase (GDH), are denoted in bold and italicized in the tables. Two proteins served as positive controls, KGA^{23,24} and GDH²⁵, both of which are known to increase in abundance during acidosis.²⁶ In this analysis, both KGA and GDH were observed to be significantly increased upon acidosis by SpC. However, only GDH was observed to significantly different by MS² TIC. Furthermore, the fold change observed for GDH by the MS² TIC method was actually a slight decrease (0.9). The significant p-value indicates that the MS² TIC values for GDH had very little variance among biological replicates. GDH is an extremely abundant protein

in these samples (mean control MS² TIC = 2.0×10^5), thus the lack of an applicable fold change is likely due to saturation of the MS² TIC signal (Table 2). While not within the significance threshold, KGA was observed to change by MS² TIC with a fold change of 1.3 (p-value = 0.08).

Western blot analysis was performed as an independent method to validate several of the proteins observed to be significantly changing in abundance in response to metabolic acidosis. Western blotting was performed with control (n=3) and acidotic (n=3) rat renal mitochondria samples (Fig. 3). Blots were probed with specific antibodies to nine proteins and normalized by probing with an antibody to a mitochondrial protein that served as a loading control (Fig. 3A). UDP-glucuronosyltransferase 1-1 (UGT1A1) was observed to be changing only by SpC (p-value = 0.0009, fold change = 4.6). However, western blotting of UGT1A1 revealed a fold change of only 1.6. The larger fold change observed by SpC could be an artificial inflation due to the detection of shared peptides from other isoforms, whereas the antibody is specific only to the 1A1 isoform. Peroxisomal multifunctional enzyme type 2 (HSD17B4), 3-ketoacyl-CoA thiolase B (ACAA1), catalase (CAT), and kidney-type glutaminase (KGA) were all detected as significantly changing only by SpC. All four of these proteins yielded statistically equivalent fold changes by western blotting with specific antibodies (Fig. 3B). Two of the proteins uniquely observed by MS² TIC were also validated by western blotting; carbonic anhydrase 5B (CA5B) and heat shock cognate 71 kDa protein (HSP7C) (Fig 3A). MS² TIC fold change for CA5B is exaggerated as this protein was not detected in control samples. CA5B narrowly fell outside the significant threshold in the SpC results, (p-value = 0.068). However, the fold change observed by SpC (5.7-fold) agrees well with the western blotting results, (3.7-fold). CA5B had a mean spectral count value of only 4.7 in the acidotic samples and 0 in the control samples which illustrates the limitation of SpC for low abundance proteins. The decrease in HSP7C, identified uniquely by MS² TIC, was also confirmed by western blotting. Of the three proteins observed by both approaches, two were further evaluated by western blotting: peroxisomal bifunctional enzyme (EHHADH) and glutamate dehydrogenase (GDH). EHHADH did not show a significant fold change by western blotting. This result may be due to the large amount of non-specific binding observed with this antibody. Statistically equivalent fold changes were observed by western blot analysis and SpC for GDH.

Previous studies have shown that SpC is not accurate for proteins at low abundance (i.e. 4 spectral counts) and MS² TIC is not accurate for proteins with 5 identified MS/MS spectra.^{15, 35, 19} To evaluate the trend within our dataset, the mean spectral counts of the triplicate acidotic samples were plotted against the calculated p-values (Fig. 4A). The majority of the data is represented by proteins with < 100 spectral counts. As the spectral counts decrease the p-values tend to be less significant, illustrating the limitation of this method for proteins at low abundance. This is further represented in the inset of figure 4A which shows a clear shift in the distribution of proteins with p-values > 0.05, above which corresponds to proteins with very low SpC values. The range of the mean normalized SpC values for proteins with p-values < 0.05 is broad, ranging from 3.8 to 715.7. Conversely, the MS² TIC values do not exhibit this trend (Fig. 4B). The scale for the MS² TIC data is notably larger compared to the SpC data (x-axis of Fig 4A vs 4B). As mentioned above, this is due to the fact that in SpC a value of 1 is assigned for each spectra whereas in MS² TIC the value for each protein corresponds the average ion signal of all the MS/MS spectra (a number much larger than 1). The difference in scale results in an increase of the quantifiable dynamic range of the MS² TIC approach.²⁰ The disadvantage of this larger scale is that saturation may occur at very high MS² TIC values. This is illustrated in Fig 4B as there are no proteins with a p-value < 0.05 at MS² TIC mean values > 3.2×10^5 . Another notable trend in the data presented in Figure 4 is that the MS² TIC data is more disperse than the SpC data. This difference highlights the superb reproducibility of SpC analysis. MS² TIC

measurements tend to be less reproducible due to the fact that in each analytical run the peptide will not be sampled at the exact same point in the chromatographic peak and this will be reflected in the MS² TIC value.

While SpC and MS² TIC are excellent methods for detecting protein differences, they are known to suffer from inaccuracy. Therefore, in this study, only a significance level threshold (p-value < 0.05) was employed. Often, however, fold changes are desired and are important in assessing biological relevance. To assess the use of fold change values in our results, fold change calculations from SpC or MS² TIC analyses were compared to the corresponding p-values from each method (Fig. 5). For both approaches a convergence to a fold change of 1 was observed with increasing p-value, this trend is expected and adds confidence to the results. Additionally, the lack of data points present at a fold change of 1 with p-values < 0.05 is also encouraging. The prominent difference in the data for the two approaches is that the MS² TIC analysis results in much larger fold change values (Fig. 5B is missing the top ten MS² TIC fold changes ranging from 20–140). The exaggerated fold changes are a result of the larger scale of MS² TIC values and thus the pseudo count approach is not as effective in dealing with zero values.

CONCLUSIONS

Label-free relative quantitation methods are extremely important in large scale proteomics experiments to further our understanding of disease states and enable biomarker discovery. In this type of study, the goal is to detect significant differences between biological states which can generate hypotheses and drive future research. Samples are often very complex and the proteins of biological interest can be at low abundance within a high background. The ability to maximally mine proteomic data to improve our ability to identify significant differences between disease states is essential. There is no current proteomic profiling strategy that is capable of identifying the entire proteome or for determining all quantitative changes between disease states. Spectral counting is advantageous in its ease of use and high reproducibility but can fail for proteins at low abundance. Conversely, MS² TIC measurements can be employed over a larger dynamic range but suffer from irreproducibility and signal saturation for highly abundant proteins. While each of these methods has specific advantages and disadvantages, together they enable a more comprehensive picture of protein abundance differences over a wide dynamic range of protein concentrations. The dual approach is straightforward and requires minimal added effort to implement. Importantly, no additional samples or mass spectrometry analyses are needed to obtain both SpC and MS² TIC values. Therefore, the added value gained by employing a dual approach cost only the additional time for data analysis. In summary, the results of this study clearly illustrate the benefit of employing a dual label-free relative quantitative approach to increase the value of a shot-gun proteomics dataset through the detection of more biologically relevant targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources:

This research was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-37124 and DK-75517 awarded to N. P. Curthoys. All mass spectrometry was performed in the Proteomics and Metabolomics Facility at Colorado State University (<http://www.pmf.colostate.edu>).

We would like to thank Dr. N. P. Curthoys for his funding support and assistance with review of this manuscript. We would like to also acknowledge Brian Searle from Proteome Software for his thoughtful review of this manuscript.

ABBREVIATIONS

MS	mass spectrometry
MS/MS	tandem mass spectrometry
TIC	total ion current

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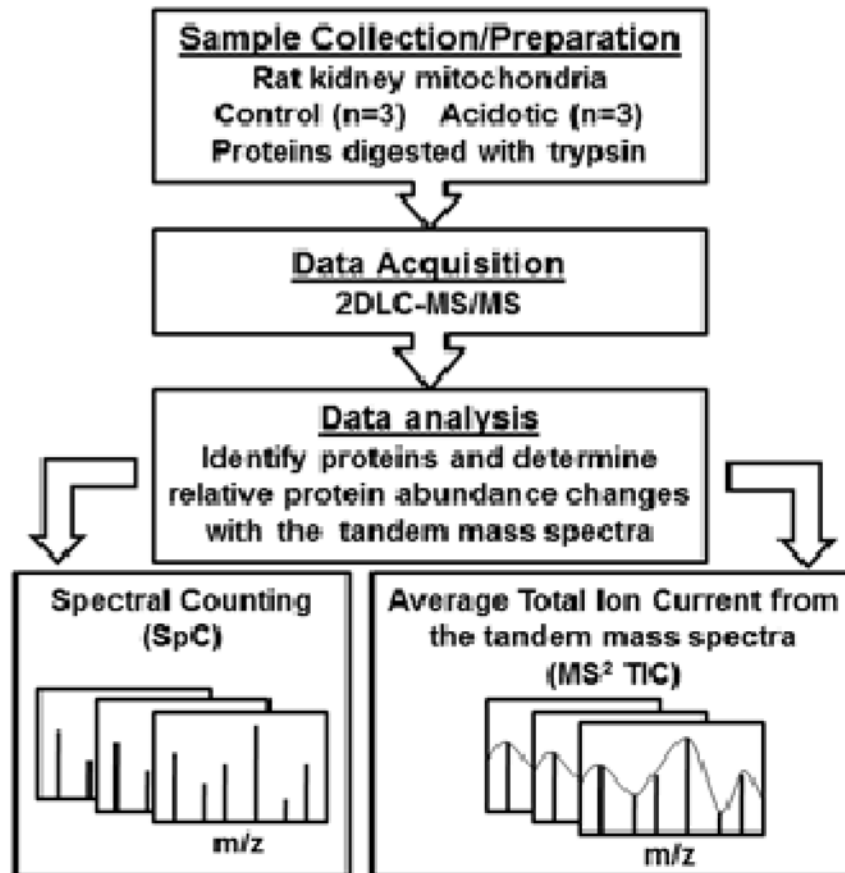


Figure 1. The experimental workflow for proteomic analysis of rat renal mitochondrial proteins. Spectral counting (SpC) and average total ion current from the tandem mass spectra (MS² TIC) was used to determine changes in protein abundance occurring in response to metabolic acidosis.

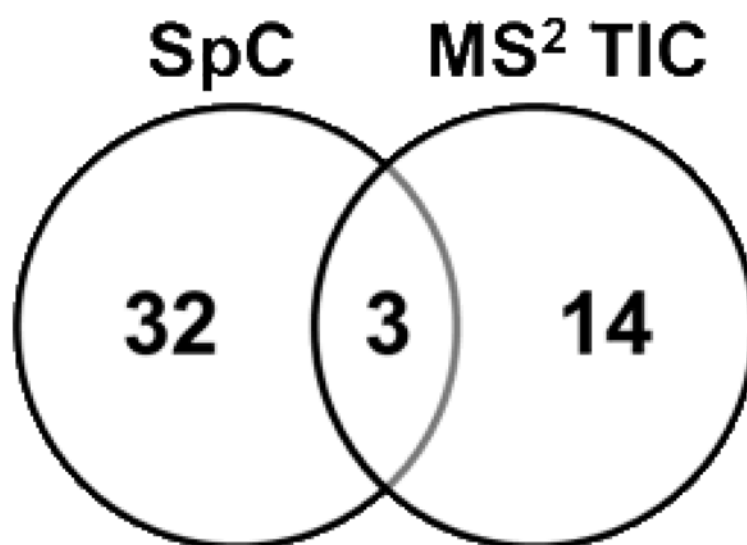


Figure 2. Venn diagram of identified proteins observed to be significantly changing (p-values < 0.05) in response to metabolic acidosis using both spectral counting (SpC) and average MS/MS total ion current (MS² TIC).

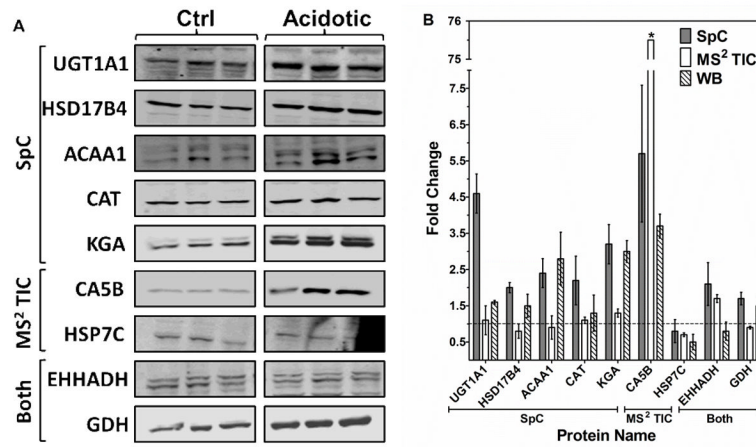


Figure 3. Western blot analysis validating proteins found to be significantly changing in response to metabolic acidosis (p-values < 0.05) by either spectral counting (SpC), average total ion current (MS² TIC) or both. A: The control (ctrl) and acidotic samples protein bands were imaged and quantified and each blot was normalized to a loading control. B: Fold changes from SpC, MS² TIC and western blotting (WB). The reported data are the mean ± the S.E. of triplicate biological samples. The * indicates error not shown for CA5B MS² TIC, ± 4.8.

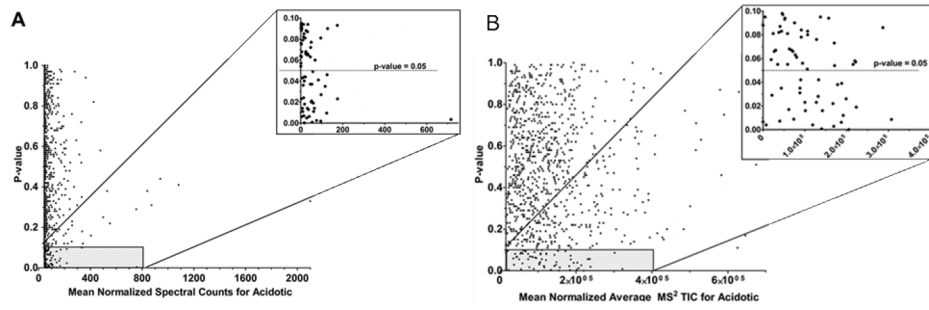


Figure 4. Quantitative value vs. p-value. A: The mean SpC of triplicate acidotic samples (x-axis) vs. p-values B: The mean MS² TIC of triplicate acidotic samples (x-axis) vs. p-values. Both insets have only the data with p-values < 0.1 (y-axis).

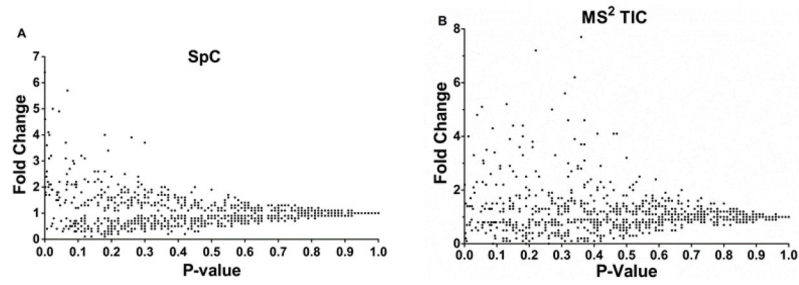


Figure 5. P-value vs. fold change. A: The p-values from SpC analysis (x-axis) and the calculated fold changes (y-axis). B: The p-values from average MS² TIC (x-axis) and the calculated fold changes (y-axis). Ten values are not plotted here; largest fold change was 137 for MS² TIC.

Table 1
 Proteins observed by SpC with significant (p-value < 0.05) abundance changes in response to metabolic acidosis

Protein name	Accession #	SpC		Mean Normalized SPC Values		Mean Normalized MS ² TIC Values	
		P-value	Fold Change	Control (S.E.)	Acidotic (S.E.)	Control (S.E.)	Acidotic (S.E.)
UDP-glucuronosyltransferase 2B15	P36511	0.00022	6.4	1.8 (1.0)	17.0 (0.7)	1.9E+05 (1.7E+05)	7.0E+04 (2.2E+04)
UDP-glucuronosyltransferase 1-1 (UGT1A1)	Q64550	0.00086	4.6	11.0 (1.8)	54.1 (4.5)	6.3E+04 (2.0E+04)	6.8E+04 (4.6E+03)
Peroxisomal multifunctional enzyme type 2 (HSD17B4)	P97852	0.0018	2.0	48.1 (3.6)	96.3 (6.3)	1.8E+06 (2.8E+04)	1.6E+06 (1.6E+04)
Dimethylglycine dehydrogenase, mitochondrial	Q63342	0.0024	1.8	40.9 (1.8)	75.0 (4.7)	1.4E+05 (1.4E+04)	1.6E+05 (2.2E+04)
Glutamate dehydrogenase 1, mitochondrial (GDH)	P10860	0.0035	1.7	431.5 (45.2)	715.7 (7.9)	2.0E+05 (7.3E+03)	1.7E+05 (3.1E+03)
3-ketoacyl-CoA thiolase B, peroxisomal (ACAA1)	F1LPD6	0.0049	2.4	11.3 (1.1)	28.4 (2.8)	4.3E+04 (1.1E+04)	3.8E+04 (6.3E+03)
Enoyl-CoA hydratase domain-containing protein 3	Q3MIE0	0.0061	3.6	0.4 (0.4)	3.8 (0.6)	1.5E+04 (1.5E+04)	6.4E+04 (2.2E+04)
Vitamin D-binding protein	P04276	0.0062	2.6	15.0 (4.4)	40.2 (1.9)	1.8E+05 (1.3E+05)	1.0E+05 (1.4E+04)
Calnexin	P35565	0.0069	0.4	35.1 (3.6)	14.8 (1.5)	6.1E+04 (1.7E+04)	7.7E+04 (9.0E+03)
Catalase (CAT)	P04762	0.0095	2.2	55.1 (12.0)	123.1 (8.3)	1.1E+05 (2.3E+04)	1.3E+05 (1.6E+04)
Fumarylacetoacetate hydrolase domain-containing protein 2	B2RYW9	0.011	2.0	29.6 (5.9)	61.1 (3.8)	1.2E+05 (2.9E+04)	1.7E+05 (7.8E+03)
Ras-related protein Rab-21	Q8AXT5	0.011	4.1	1.5 (0.8)	9.5 (1.6)	3.8E+04 (2.8E+04)	5.5E+04 (1.1E+04)
Epoxide hydrolase 1	P07687	0.013	3.1	11.1 (3.3)	36.3 (4.9)	2.2E+05 (6.1E+04)	2.2E+05 (3.4E+04)
Cytochrome b5	P00173	0.014	1.7	37.5 (5.8)	64.7 (2.8)	2.2E+05 (1.3E+04)	2.6E+05 (2.2E+04)
Peroxisomal bifunctional enzyme (EHHADH)	P07896	0.014	2.1	14.3 (3.5)	31.5 (2.0)	8.2E+04 (1.7E+04)	2.2E+05 (1.7E+04)
Uncharacterized protein	D4A0Y1	0.014	4.0	1.2 (0.7)	7.7 (1.4)	1.2E+04 (6.8E+03)	3.0E+04 (2.3E+03)
Ectonucleoside triphosphate diphosphohydrolase 5	Q6P6S9	0.018	2.1	8.0 (0.8)	18.2 (2.5)	6.6E+04 (8.3E+03)	4.4E+04 (4.2E+03)
Kidney-type glutaminase (KGA)	P13264	0.019	3.2	17.7 (2.1)	69.3 (10.7)	0.2E+04 (3.1E+03)	1.1E+05 (1.0E+04)
4F2 cell-surface antigen heavy chain	Q794F9	0.02	0.5	105.6 (13.0)	50.5 (6.8)	1.3E+05 (2.7E+03)	1.2E+05 (2.1E+04)
Peroxisomal acyl-coenzyme A oxidase 1	P07872	0.021	1.9	26.2 (6.8)	51.4 (0.5)	5.1E+04 (5.1E+03)	7.1E+04 (1.6E+04)
Cytochrome P450 4A2	P20818	0.023	1.9	91.2 (22.0)	175.1 (7.7)	3.8E+04 (3.8E+03)	4.7E+04 (9.3E+03)
Filamin-B	D3ZD13	0.023	0.6	4.2 (0.5)	2.1 (0.3)	4.0E+04 (1.2E+04)	3.6E+04 (1.1E+04)
Protein AMBP	Q64240	0.024	5.0	2.0 (0.9)	13.8 (3.2)	8.1E+04 (5.7E+04)	7.2E+04 (1.9E+04)
ATP synthase-coupling factor 6, mitochondrial	P21571	0.027	0.8	115.6 (4.4)	96.9 (3.3)	2.7E+05 (2.9E+04)	2.4E+05 (1.3E+04)
Enoyl-CoA delta isomerase 1, mitochondrial	P23965	0.035	1.5	76.7 (6.5)	116.2 (10.8)	1.0E+05 (2.0E+04)	1.3E+05 (9.5E+03)
Protein FAM151A	Q642A7	0.036	0.4	32.8 (6.3)	11.7 (2.5)	5.2E+04 (1.1E+04)	9.1E+04 (2.2E+04)

Protein name	Accession #	SpC		Mean Normalized SPC Values		Mean Normalized MS ² TIC Values	
		P-value	Fold Change	Control (S.E.)	Acidotic (S.E.)	Control (S.E.)	Acidotic (S.E.)
ATP-binding cassette sub-family D member 3	P16970	0.037	1.8	29.3 (6.3)	52.5 (4.0)	8.1E+04 (2.5E+04)	1.1E+05 (8.4E+03)
Probable D-lactate dehydrogenase, mitochondrial	F1LVD7	0.037	1.7	27.0 (4.0)	45.7 (4.5)	3.0E+05 (8.6E+04)	2.4E+05 (3.1E+04)
GrpE protein homolog 1, mitochondrial	P97576	0.04	1.5	10.2 (0.9)	16.3 (1.8)	2.3E+05 (4.7E+04)	2.8E+05 (6.9E+04)
<i>Apollipoprotein A-IV</i>	P02651	0.041	1.8	46.3 (10.0)	83.6 (7.6)	1.6E+05 (1.5E+03)	2.0E+05 (1.3E+04)
All-trans-13, 14-dihydroretinol saturase, isoform CRA_b	G3V7V6	0.043	4.9	0.7 (0.4)	7.5 (2.3)	5.8E+04 (3.5E+04)	1.1E+05 (7.7E+03)
NADPH--cytochrome P450 reductase	P00388	0.044	1.6	13.2 (1.4)	21.6 (2.6)	8.5E+04 (1.2E+04)	8.7E+04 (7.1E+03)
Ribonuclease 4	O55004	0.044	2.2	7.5 (1.4)	17.7 (3.2)	1.4E+05 (3.8E+04)	1.6E+05 (7.5E+03)
Electron transfer flavoprotein subunit beta	Q68FU3	0.046	1.3	92.3 (9.3)	125.5 (6.5)	1.6E+05 (2.1E+04)	1.7E+05 (7.2E+03)
Lon protease homolog, mitochondrial	Q924S5	0.049	0.7	86.5 (10.1)	57.5 (2.7)	8.6E+04 (1.7E+04)	6.2E+04 (4.2E+03)

Bold & italicized are common to both methods.

Table 2

Proteins observed by MS² TIC with significant p-value < 0.05) abundance changes in response to metabolic acidosis

Protein name	Accession #	MS ² TIC		Mean Normalized MS ² TIC Values		Mean Normalized SpC Values	
		P-Value	Fold Change	Control (S.E.)	Acidotic (S.E.)	Control (S.E.)	Acidotic (S.E.)
Carbonic anhydrase 6B, mitochondrial (CA6B)	Q88HG8	9.9E-06	76.6	0.0E+00 (0.0E+00)	2.1E+06 (1.3E+04)	0.0 (0.0)	4.7 (1.8)
RCG41951, isoform CRA_a	D3ZCZ9	0.00073	2.2	6.2E+04 (6.5E+03)	1.4E+05 (5.3E+03)	15.2 (5.1)	21.3 (1.3)
Histone H4	P62804	0.0028	0.4	4.2E+05 (2.6E+04)	1.7E+05 (2.9E+04)	6.6 (22)	9.5 (1.2)
Tropomyosin alpha-3 chain	F1LRP5	0.004	0.2	5.8E+04 (4.0E+03)	7.5E+03 (7.5E+03)	17.7 (8.9)	0.3 (0.3)
Saccharopine dehydrogenase-like oxidoreductase	Q6AY30	0.0072	0.7	5.9E+04 (2.0E+03)	4.0E+04 (3.1E+03)	25.1 (9.4)	17.6 (1.4)
Uncharacterized protein	E9PT15	0.0086	1.4	2.2E+05 (1.3E+04)	3.1E+05 (1.2E+04)	71.9 (7.1)	92.0 (10.5)
Heat shock cognate 71 kDa protein (HSP7C)	P63018	0.016	0.7	2.0E+05 (4.3E+03)	1.4E+05 (1.2E+04)	94.3 (25.0)	73.1 (3.8)
EH domain-containing protein 1	Q64IZ6	0.017	0.8	8.2E+04 (3.7E+03)	6.2E+04 (3.7E+03)	57.3 (19.8)	40.4 (2.1)
<i>Peroxisomal bifunctional enzyme (EHHADH)</i>	P07898	0.019	1.7	8.2E+04 (1.7E+04)	2.2E+06 (1.7E+04)	14.3 (3.6)	31.6 (2.0)
<i>Glutamate dehydrogenase 1, mitochondrial (GDH)</i>	P10860	0.022	0.9	2.0E+05 (7.3E+03)	1.7E+05 (3.1E+03)	431.5 (45.2)	715.7 (7.9)
Tubulin beta-5 chain	P69897	0.023	1.4	7.0E+04 (5.8E+03)	1.0E+05 (5.9E+03)	106.4 (30.0)	89.7 (8.5)
<i>Apolipoprotein A-IV</i>	P02651	0.026	1.3	1.5E+05 (1.5E+03)	2.0E+05 (1.3E+04)	46.3 (10.0)	83.6 (7.6)
Xaa-Pro aminopeptidase 1	O54975	0.028	2.1	6.2E+04 (1.8E+04)	1.3E+05 (1.0E+04)	6.9 (23)	7.9 (3.8)
Glutaryl aminopeptidase	P50123	0.035	0.6	1.4E+05 (1.4E+04)	8.8E+04 (9.6E+03)	38.8 (10.4)	20.8 (8.0)
Sulfate anion transporter 1	P45380	0.038	2.3	7.7E+04 (1.1E+04)	1.8E+05 (3.1E+04)	6.8 (0.9)	3.9 (1.1)
NADH dehydrogenase 1 alpha subcomplex. 8	D4A311	0.042	0.5	2.7E+05 (4.7E+04)	1.3E+05 (9.1E+03)	41.9 (10.3)	49.1 (4.4)
Triosephosphate isomerase	P48600	0.042	0.8	1.1E+06 (8.4E+03)	9.0E+04 (3.3E+03)	64.7 (9.0)	32.1 (2.9)

Bold & italicized are common to both methods.