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Systematic Comparison of Label-Free, Metabolic Labeling, and Isobaric Chemical Labeling for Quantitative Proteomics on LTQ Orbitrap Velos

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Abstract

A variety of quantitative proteomics methods have been developed, including label-free, metabolic labeling, and isobaric chemical labeling using iTRAQ or TMT. Here, these methods were compared in terms of the depth of proteome coverage, quantification accuracy, precision, and reproducibility using a high-performance hybrid mass spectrometer, LTQ Orbitrap Velos. Our results show that (1) the spectral counting method provides the deepest proteome coverage for identification, but its quantification performance is worse than labeling-based approaches, especially the quantification reproducibility; (2) metabolic labeling and isobaric chemical labeling are capable of accurate, precise, and reproducible quantification and provide deep proteome coverage for quantification. Isobaric chemical labeling surpasses metabolic labeling in terms of quantification precision and reproducibility; (3) iTRAQ and TMT perform similarly in all aspects compared in the current study using a CID-HCD dual scan configuration. Based on the unique advantages of each method, we provide guidance for selection of the appropriate method for a quantitative proteomics study.

Introduction

Quantitative proteomics measures abundance changes of many proteins among multiple samples in a high-throughput manner [1]. Results from such measurements provide information on how biological systems respond to environmental perturbations at a genomic scale. A number of methods have been developed for quantitative proteomics to obtain high proteome coverage, accurate quantification, and wide applicability to different types of samples [2]. In proteomics analysis based on 2-dimensional gel electrophoresis (2D-GE) [3], quantification is achieved by measuring staining intensities of protein spots. To eliminate gel-to-gel variability, proteomes under comparison can be labeled separately using different fluorescent cyanine dyes (Cy2, Cy3, and Cy5) and then combined for 2D-GE analysis [4]. However, both identification and quantification are difficult for gel spots containing multiple co-migrating proteins [5]. Only one of those co-migrating proteins may be identified in such a gel spot and that protein may not be the one responsible for the differential expression. In addition, the capability of 2D-GE proteomics is also limited by the number of quantifiable proteins in a gel, a bias against membrane proteins, and a low sample throughput [1].

In the shotgun proteomics approach, proteins are typically digested using proteases into peptides which are then analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [6]. Without using any isotopic or chemical modification of proteins or peptides, label-free quantification can be achieved by correlating protein abundance with either mass spectrometric signal intensities of peptides [7] or the number of MS/MS spectra matched to peptides and proteins (spectral counting) [8]. Label-free quantification is widely used because it allows simultaneous identification and quantification of proteins without a laborious and costly process of introducing stable isotopes into samples and this approach is applicable to samples from any source. However, because samples to be quantified are prepared and measured

separately, label-free approaches have limited quantification performance in terms of accuracy, precision and reproducibility.

To improve quantification performance, many approaches were developed based on stable isotope labeling, including metabolic labeling [9], enzymatic labeling [10], and chemical labeling [11]. In metabolic labeling, stable heavy isotopes are incorporated into proteins by growing cells in controlled media containing an ^{15}N-enriched nitrogen source [12] (^{15}N labeling) or isotopically labeled essential amino acids (stable isotope labeling by amino acids in cell culture or SILAC [13]). Metabolic labeling allows samples grown in different states to be combined at the cell level. Therefore, any bias in the downstream sample preparation and measurement would alter protein abundances from different samples to the same extent, making their ratios relatively unchanged. However, many biological systems are not amenable to efficient metabolic labeling, such as natural microbial communities [14]. To overcome this, chemical or enzymatic methods have been developed to label proteins or peptides using different isotopic tags. For example, after cell lysis, extracted proteins can be labeled using isotope-coded affinity tags (ICAT) [11]. After protein digestion, peptides can be labeled enzymatically at the C-terminus using $H_2{}^{18}O$ [10]. Peptides can also be labeled on the primary amine group at the N-terminus and lysine side chain using reductive dimethylation (ReDi) [15]. In proteomics measurements based on these stable-isotope labeling strategies, the abundance ratios of mass-different isotopic variants of peptides are determined using their signal intensities in full parent ion scans of the LC-MS/MS analysis. Abundance ratios of peptides are then used to infer abundance ratios of their parent proteins.

Recently, two similar isobaric chemical labeling methods, Isobaric Tag for Relative and Absolute Quantification (iTRAQ) [16] and Tandem Mass Tag (TMT) [17], have become increasingly popular for quantitative proteomics. After proteolysis, samples are labeled separately with different isotopic variants of iTRAQ or TMT and are then combined for LC-MS/MS analysis. Both iTRAQ and TMT tags contain three functional parts: a reporter ion group, a mass

normalization group and an amine-reactive group. The amine-reactive group specifically reacts with N-terminal amine groups and epsilon-amine groups of lysine residues to attach the tags to peptides. The mass normalization groups balance the mass difference among the reporter ion groups such that different isotopic variants of the tag have the same mass. Peptides labeled with different variants of the tag are indistinguishable in full scans, which prevents increasing the full-scan complexity after mixing multiple samples. In MS/MS scans, reporter ions of different masses are dissociated from isolated peptide species. The mass of a reporter ion is associated with a specific variant of the tag and the relative intensity of the reporter ions measures the relative abundance of the peptide labeled with that specific tag variant. 6-plex TMT [18] and 8-plex iTRAQ [19] allow comparing up to 6 and 8 samples in a single LC-MS/MS analysis, respectively. Multiplexing is a unique capability of iTRAQ and TMT in comparison to the other labeling techniques.

Each of the described methods has its advantages and disadvantages for quantitative proteomics. A comparison of SILAC and spectral counting showed that spectral counting provided less precise quantification to proteins with low spectral counts [20]. A comparison of $^{14}N/^{15}N$ metabolic labeling with spectral counting showed that spectral counting was less sensitive to detecting small fold changes [21]. iTRAQ was also compared to a label-free quantification method based on normalized chromatographic peak intensity [22]. While the number of identified proteins and reproducibility were comparable between these two methods, proteome coverage was significantly higher in the label-free method. To date, no study has systematically compared label-free, metabolic labeling, and isobaric chemical labeling with iTRAQ or TMT using the same analytical platform.

In this study, performances of spectral counting, $^{14}N/^{15}N$ metabolic labeling, iTRAQ and TMT were benchmarked using standard proteome samples prepared from a model microorganism, *Pseudomonas putida* F1 [23] (Figure 1). *P. putida* F1 is a gram negative soil microbe, known for its

diverse metabolism and ability to degrade aromatic hydrocarbons. Its unique bioremediation potential is frequently exploited for remedying contaminated soils. Measurements for all four methods were performed using the LTQ Orbitrap Velos [24]. The higher-energy collisional dissociation (HCD) capability and the improved ion extraction efficiency of LTQ Orbitrap Velos enabled excellent measurement of iTRAQ- or TMT-labeled samples.

Experimental Section

Chemicals and reagents. HPLC-grade water and acetonitrile (ACN) were obtained from Burdick & Jackson (Muskegon, MI), and 98% formic acid from EM Science (Darmstadt, Germany). iTRAQ 4-plex and TMT duplex reagents were purchased from Applied Biosystems and Thermo Scientific, respectively. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

Strain, media, and growth conditions. *Pseudomonas putida* strain F1 (ATCC 700007) was grown aerobically at 30 °C with vigorous shaking (200 rpm) in M9 minimal medium [2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 1X M9 salts (5X M9 salts contain per liter: 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl (normal NH_4Cl for unlabeled medium and 98%-enriched $^{15}NH_4Cl$ for ^{15}N-labeled medium), 64 g $Na_2HPO_4 \cdot 7H_2O$)] supplemented with 50 mM (final concentration) of glucose [23]. The M9 minimal medium was sterilized by autoclaving and 1 M glucose stock solution was sterilized by passing through a 0.2 µm filter (Nalgene). Three cell cultures were grown identically. The unlabeled medium was used for the culture 1 and 2 and the ^{15}N-enriched medium was used for the culture 3 (Figure 1). Cells were harvested from the three cultures at the mid-log phase of growth (OD_{600}~0.4).

Proteome sample preparation. For label-free quantification, two standard samples were prepared using 1 g of cell pellet from the culture 1 and 1 g of cell pellet from the culture 2. For

metabolic-labeling quantification, one standard sample was prepared by mixing 1 g of unlabeled cell pellet from the culture 1 and 1 g of ^{15}N-labeled cell pellet from the culture 3. For TMT or iTRAQ quantification, two peptide samples were prepared separately using 1 g of cell pellets from the cultures 1 and 2, respectively, and were mixed after labeling. For each quantification method, all quantified proteins were expected to have an abundance ratio of 1:1. All samples were prepared identically in parallel. Briefly, cells were lysed by sonication in 6 M guanidine and 10 mM dithiothreitol (DTT). The extracted proteins were precipitated by chilled acetone. Protein pellets were obtained by centrifugation (15000 rpm), air dried and then re-solubilized in Triethylammonium Bicarbonate (TEAB) buffer. Protein concentration was measured by Bicinchoninic Acid (BCA) (Thermo Scientific) assay following the manufacturer's protocol. Sequencing grade trypsin (Promega, Madison, WI) was added at 1:100 (wt:wt) into proteins in TEAB buffer supplemented with 10 mM $CaCl_2$(final concentration). The first digestion was run overnight at 37 °C and, after adding additional trypsin at 1:100 (wt:wt) into proteins, the second digestion was run for 5 hrs at 37° C. Finally, the samples were reduced with 10 mM DTT for 1 hr at 60 °C and desalted using C18 solid-phase extraction (Sep-Pak Plus, Waters, Milford, MA). BCA assay was conducted to determine peptide concentration. Label-free samples and ^{14}N/^{15}N-labeled samples were stored at -80° C before LC-MS/MS.

The two iTRAQ samples, each containing 100 µg of peptides, were labeled using iTRAQ-116 and iTRAQ-117 following the manufacturer's standard protocol. The two samples were then mixed, yielding the standard sample for iTRAQ. Similarly, the two TMT samples, each containing 100 µg of peptides, were labeled using TMT-126 and TMT-127 following the manufacturer's protocol. The two samples were then mixed, yielding the standard sample for TMT.

2D-LC-MS/MS measurements. In each measurement, 50 µg of peptides from a standard sample were loaded offline into a 150-µm-I.D. 2D back column (Polymicro Technologies) which

contained 4 cm of C18 reverse phase (RP) resin (Luna, Phenomenex) and 4 cm of strong cation exchange (SCX) resin (Luna, Phenomenex). The back column was connected to a 15-cm-long 100-µm-I.D. C18 RP PicoFrit column (New Objective) and placed in-line with a U3000 quaternary HPLC (Dionex, San Francisco, CA). Before SCX separation, a 1-hr RP gradient from 100% Solvent A (95% H_2O, 5% ACN, and 0.1% formic acid) to 100% Solvent B (30% H_2O, 70% ACN, and 0.1% formic acid) was configured to move peptides from C18 resin to SCX resin in the back column. The SCX LC separation was performed with eleven salt pulses containing increasing concentrations of ammonium acetate. Each salt pulse was followed by a 2-hr reverse phase gradient from 100% Solvent A to 60% Solvent B. The LC eluent was directly nanosprayed into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) with an ionization voltage of 4 KV. During the chromatographic separation, the LTQ Orbitrap Velos was operated in a data-dependent mode and under direct control of the Xcalibur software (Thermo Scientific). The MS data were acquired using the following parameters: ten data-dependent collisional-induced-dissociation (CID) MS/MS scans per every full scan in label-free; six data-dependent CID MS/MS scans per every full scan in metabolic labeling; and four data-dependent CID-HCD dual MS/MS scans [25] per every full scan in iTRAQ and TMT; CID scans were acquired in LTQ with two-microscan averaging; full scans and HCD scans were acquired in Orbitrap at resolution 30,000 and 7,500, respectively, with two-microscan averaging; 35% normalized collision energy (NCE) in CID and 55% NCE in HCD; ±1.5 Da isolation window; dynamic exclusion enabled with ±1.5 Da exclusion window. In CID-HCD dual scan, each selected parent ion was first fragmented by CID and then by HCD.

Bioinformatics. All MS/MS spectra were searched using SEQUEST [26] against the *P. putida* F1 genome database (available at http://compbio.ornl.gov/pseudomonas_putida_F1/chromate_response/databases/) containing in FASTA format a total of 5251 predicted proteins and 44 common contaminants (trypsin, keratin,

etc.). The reversed sequences of all proteins were appended into the database for calculation of false discovery rate (FDR) [27]. The SEQUEST searches for label-free samples and $^{14}N/^{15}N$-labeled samples were performed as described previously [23]. Two SEQUEST searches were performed for each iTRAQ and TMT run. The first search used static modification at the N-terminus and dynamic modification at the lysine residue by the labeling reagents. The second search used only dynamic modification at the lysine residue. The output data files were then filtered and sorted using the DTASelect v1.9 [28] algorithm as described previously [23].

For label-free quantification, the raw spectral counts calculated by DTASelect for identified proteins were normalized using the following formula:

$$N_i = R_i \cdot \frac{\bar{C}}{C_i},$$

where N_i and R_i are the normalized and raw spectral counts of a protein in run i, respectively; C_i is the total spectral count of run i; and $\bar{C}$ is the averaged total spectral count of all the runs under comparison. The scaling factor, $\frac{\bar{C}}{C_i}$, was used to normalize total spectral count of each run to the same to reduce run-to-run variability. $^{14}N/^{15}N$ quantification was performed using the ProRata [29] program as described (freely available at http://code.google.com/p/prorata/). Perl scripts were developed to process iTRAQ and TMT datasets for protein quantification. Briefly, all LC-MS/MS datasets from iTRAQ and TMT experiments were converted from the Xcalibur Raw file format to the MS2 flat file format using the Raxport [30] program freely available at http://code.google.com/p/raxport/. In the CID-HCD dual scan configuration, peptide identification can be obtained from the CID scan, the linked HCD scan, or both. Reporter ions for all peptide identifications were extracted from small windows (±0.02 Da) around their expected m/z in the HCD scan. If multiple peaks were found within the accepted m/z window of a reporter

ion, the one with the highest intensity was considered to represent the reporter ion. The total intensity at a reporter ion channel for a protein was calculated as the sum of this reporter ion's intensities from all constituent unique peptides from this protein [31]. The abundance ratio of a protein was estimated using the ratio between the protein's total intensities in different reporter ion channels.

Results and Discussion

Comparison of protein and peptide identification results

The results of protein identifications from label-free, metabolic labeling, and isobaric chemical labeling are summarized in Table 1. All the identified proteins from each run are reported in Supplemental Table 1. A total of 1980 unique proteins were identified using the label-free method (on average approximately 1600 non-redundant proteins from a run, FDR = 2%). 79% of all identified proteins in the duplicate runs of a sample were identified reproducibly in both duplicate runs (Supplemental Figure 1A). A total of 1606 unique proteins were identified using the metabolic labeling method with 77% identification reproducibility between duplicate runs (FDR = 3%) (Supplemental Figure 1B). 1473 unique proteins were detected from the iTRAQ-labeled sample (FDR = 2%) and 1404 in the TMT-labeled sample (FDR = 3%). 73% of proteins were identified reproducibly between duplicate runs in iTRAQ (Supplemental Figure 1C) and 76% in TMT (Supplemental Figure 1D). This shows that the label-free method had the highest number of protein identifications and provided the deepest coverage of the genome (~30%). Identification reproducibility between duplicates was similar among all four methods.

Different data acquisition schemes were used for label-free, metabolic labeling, and isobaric chemical labeling in current study. Every full scan was followed by ten data-dependent CID MS^2 scans in the label-free analysis, which generated the highest number of identified

peptides and proteins. Because in metabolic labeling proteins were quantified using full scans, six data-dependent CID MS^2 scans per full scan were configured to provide more frequent full scan acquisition and better reconstruction of chromatographic peaks of peptides. The sample complexity in full scans was doubled as a result of mixing an unlabeled proteome with a ^{15}N-labeled proteome. Because many peptides were identified redundantly in both isotopic variants, although more spectra were identified in the metabolic labeling analysis than in the label-free analysis, fewer peptides and proteins were identified and the average sequence coverage of proteins was not increased. For iTRAQ and TMT analysis, every full scan was followed by four CID-HCD dual MS^2 scans, in which a selected parent ion was first fragmented by CID for peptide identification and then by HCD for quantification. HCD offers higher fragmentation efficiency and lower minimum m/z detection limit than CID, which enables measurement of reporter ions in Orbitrap analyzer with high signal-to-noise ratio. However, because of the extra time needed for HCD analysis, the duty cycle of MS^2 acquisition was significantly lower in the CID-HCD dual-scan configuration than the CID-only configuration used for the other analyses. Furthermore, previous studies have shown that the presence of fragment ions as a result of losing isobaric tags from precursor ions complicates interpretation of spectra by database searching algorithms [32]. Therefore, fewer peptides and fewer proteins were identified in isobaric chemical labeling than in label-free and metabolic labeling (Table 1). Similar protein identification results were observed between iTRAQ and TMT.

Because HCD spectra can be used for both peptide identification and quantification, TMT and iTRAQ samples can be analyzed using only HCD [33]. We found that only less than 30% of identified spectra were from HCD fragmentation. Less than 10% of those identified HCD spectra have a paired CID spectrum that did not identify a peptide; whereas approximately 60% of identified CID spectra have a paired HCD spectrum that did not identify a peptide. This indicates the value of CID for peptide identification. The duty cycle of the CID-HCD

configuration was not significantly lower than the HCD-only configuration, because the acquisition time for CID coupled with ion-trap detection is only a fraction of the acquisition time for HCD coupled with Orbitrap detection in the dual scan.

Isobaric mass tags were chemically linked to N-terminus amine groups and the epsilon-amine group of lysine. In one database search, derivatization of the N-terminus was set as a static modification and dynamic modification was set at lysine residue. >98% of lysine residues in the identified peptides were labeled, indicating high labeling efficiency of lysine in sample preparation. A separate search for peptides with an unmodified N-terminus using dynamic modification at lysine identified only a few hundred peptides with a greater than 50% FDR, which suggests a high labeling efficiency of the N-terminus by iTRAQ and TMT.

Ross et al observed that the ratio of Lys-terminated peptides to Arg-terminated peptides (Lys:Arg peptide ratio) increased from 0.79 in an unlabeled sample to 0.98 in an iTRAQ labeled sample [16]. However, in this study, the Lys:Arg peptide ratios from TMT and iTRAQ were not significantly higher than those from label-free or metabolic labeling (Table 1). An expected Lys:Arg peptide ratio of 0.50 (170,662 Lys-ending peptide and 342,497 Arg-ending peptides.) was calculated based on *in silico* digestion[34] of the *P. putida* F1 proteome. The observed Lys:Arg peptide ratios in all runs were higher than the expected ratio.

Comparison of protein quantification results

Standard samples were prepared for the quantitative proteomics methods under comparison such that every protein was expected to have an abundance ratio of 1:1 (Figure 1). The measured abundance ratios of peptides and proteins were transformed to $\log_2$ scale ($\log_2$ratio). Protein quantification results from each quantitative proteomics method are summarized in Table 2. All quantified proteins and peptides from each run are reported in the Supplemental Table 2 and Table 3, respectively. Supplemental Figure 2 shows that the majority of spectral counting variability stemmed from proteins with low spectral counts. Therefore, a minimum spectral count

cutoff of four was used to filter out proteins with poor quantification precision [35]. As a result, although more proteins were identified using the label-free approach than labeling-based approaches, fewer proteins were precisely quantified.

For iTRAQ and TMT measurements, we examined the relationship between the reporter ion intensity and the quantification accuracy and precision of peptides. Log_2ratio of peptides were plotted against reporter ion intensity in log_2 scale (log_2intensity) (Figure 2A and 2B). For both iTRAQ and TMT, most peptides had reporter ion intensities greater than 2^{10} and were quantified accurately. The log_2ratios of peptides measured by iTRAQ have greater spread than those measured by TMT at log_2intensity below 10 (Figure 2A and 2B), indicating that the observed TMT ratios were slightly more precise. The median of peptide log_2ratios was slightly closer to 0 in the TMT runs than in the iTRAQ runs (Table 2), suggesting that TMT ratios were slightly more accurate. Therefore, TMT may have slightly better quantification performance than iTRAQ at the peptide level. However, there was little difference at the protein level (Table 2). To assess quantification accuracy and precision at different reporter ion intensity ranges, peptides were binned by their reporter ion intensities and the median and median absolute deviation (MAD) of log_2ratios in each intensity bin were calculated (Figure 2C). The quantification precision as measured by MAD was consistently maintained at ~0.2 across the entire range of reporter ion intensities. Karp *et al* observed that the quantification variability was higher at lower reporter ion intensities in iTRAQ measurements[36]. This discrepancy may be due to different instruments and data acquisition schemes used in the two studies. The quantification bias as measured by the deviation of the median from the expected value, 0, decreased as the reporter ion intensity increased. The quantification bias for low-intensity peptides could stem from the background noise in the detection of their reporter ions. Thus, peptides with higher reporter ion intensities should be given higher weight when used to calculate a protein's relative abundance. To be general to comparisons involving more than two samples, let us represent a protein's relative abundance in sample *x* as the percentage of the protein's quantity in sample *x* out of the protein's

total quantity from all mixed samples or $x\%$. Suppose this protein has n quantified peptides. $x\%$ can be calculated as:

$$x\% = \sum_{i=1}^{n} \frac{x_i}{P_i} \cdot \frac{P_i}{T},$$

where x_i is the reporter ion intensity of peptide i at the reporter ion channel corresponding to sample x, P_i is the total reporter ion intensity of peptide i from all channels, and T is the sum of the total reporter ion intensities of all peptides from this protein. In this formula, the relative reporter ion intensity of a peptide at a channel, $\frac{x_i}{P_i}$, is simply weighted by its total ion intensity, P_i, when it is pooled together with other peptides to calculate a protein's relative abundance. This is mathematically equivalent to the summing method previously described [31]:

$$x\% = \sum_{i=1}^{n} \frac{P_i}{T} \cdot \frac{x_i}{P_i} = \frac{\sum_{i=1}^{n} x_i}{T}.$$

In this study, abundance ratios of proteins were calculated using this approach for TMT and iTRAQ. As a result, the overall quantification accuracy and precision were significantly better for proteins than for peptides.

Quantification precision of proteins by the four quantitative proteomics methods was compared using MAD of protein $\log_2$ratios and the percentage of proteins within 2-fold abundance change (Table 2). The performance metrics were highly reproducible between the two technical replicates of every method. To examine how the measured protein and peptide abundance ratios from each method were distributed, density plots were generated for the set of $\log_2$ratios from each method, both at the protein level and at the peptide level (Figure 3). The distributions from iTRAQ and TMT experiments were narrowest, indicating the highest quantification precision. Together, our data demonstrates that iTRAQ and TMT provided the most precise measurements and will be more sensitive for detecting protein expression with small fold changes. Metabolic labeling was able to yield accurate quantification, however the

measurement variability was relatively wider than iTRAQ and TMT. Although the spectral counting method was the least precise among the compared methods, reasonable quantitative results can be still obtained.

We finally examined quantification reproducibility of each method across technical replicates. Protein $\log_2$ratios from duplicate measurements of each method were plotted on a two-dimensional histogram (Figure 4). Correlation between protein $\log_2$ratios of the technical duplicates was also the lowest in the spectral counting analysis (R^2 = 0.2) (Figure 4A). Note that spectral counts of proteins from two technical replicates of a culture are relatively reproducible: R^2 = 0.86 for culture 1 and R^2 = 0.87 for culture 2 (Supplemental Figure 2). Quantification reproducibility was significantly improved in labeling-based approaches: R^2 = 0.77 for metabolic labeling (Figure 4B) and R^2 = 0.87 for iTRAQ and TMT (Figure 4C and 4D). Note that biological variability was observed to be more significant than technical variability in comparison of different biological samples. Therefore, regardless of the quantification method used, it is important to use not only technical replication but also biological replication for statistical assessment in biological studies[37,38].

Considerations in method selection for a quantitative proteomics study

In label-free quantification, each sample of interest must be prepared and analyzed by LC-MS/MS separately. The semi-random-sampling nature of the peptide identification process in a shotgun proteomics run also contributes to the variability of spectral counting for protein quantification. Therefore, relatively poor quantification results were observed with the spectral counting method. Several alternative MS/MS acquisition methods have been developed, which could overcome this limitation. Venable *et al* introduced a data-independent acquisition method based on sequential isolation and fragmentation of a series of pre-determined precursor windows [39]. Carvalho *et al* extended this method and developed an algorithm to identify multiplexed spectra acquired with CID and electron transfer dissociation [40]. In the MS^E approach, a quadrupole time-of-flight mass spectrometer was used to fragment all precursor ions in an

elevated-energy mode [41]. These data-independent methods will probably increase the reproducibility of label-free quantification. Alternative data analysis methods have also been developed to improve label-free quantification. For example, chromatographic peak areas of peptides, instead of spectral counts, can be used as the measure of protein abundance for quantification [35]. The normalized spectral index (SI_N) method estimates protein abundance by combining spectral counts and total ion intensity of MS/MS spectra [42].

In contrast to label-free quantification in terms of sample preparation, metabolic labeling allows mixing of samples at the very beginning of preparation. Samples representing two states are prepared and measured together, which minimizes potential bias in these processes. The relative abundance ratio of a protein between samples is maintained. Thus, accurate and reproducible quantification results can be obtained from metabolic labeling.

In iTRAQ and TMT analysis, samples from different conditions are processed separately until peptides are generated and labeled with different tags. After that, these samples are pooled for subsequent LC-MS/MS measurement. HCD provides efficient ion extraction and fragmentation for generation of reporter ions, allowing detection of reporter ions with high signal-to-noise ratio in Orbitrap analyzer. In comparison to metabolic labeling, MS detection of reporter ions in an Orbitrap MS2 scan may be better for quantifying a peptide than detection of precursor ions in a series of Orbitrap MS1 scans. Thus, although TMT and iTRAQ require samples to be mixed at a later sample preparation stage than metabolic labeling, they produced better overall quantification results.

The comparison results provided guidance for choosing an appropriate approach for a proteomics experiment. The label-free method has the largest dynamic range for protein identification; however, high spectral counts are required for reliable quantification. In addition, special care is necessary to minimize sample-to-sample variability during sample preparation and measurement. Both metabolic labeling and isobaric chemical labeling provide accurate, precise, and reproducible quantification for many proteins, but each has advantages and disadvantages.

Metabolic labeling is ideal for samples that need to undergo extensive preparation steps at the protein level, such as fractionation and enrichment, which may introduce a significant amount of error without pooling samples together. However, metabolic labeling is feasible only for selected microorganisms and cell cultures. The unique advantage of iTRAQ and TMT is the capability to multiplex more than two samples in a measurement. This not only saves instrument time but also simplifies experimental design. However, iTRAQ and TMT require advanced MS instruments, such as Q-TOF and LTQ Orbitrap Velos.

Conclusion

In this study, four quantitative proteomic approaches, label-free, metabolic labeling, and isobaric chemical labeling by iTRAQ or TMT, were compared using an LTQ Orbitrap Velos mass spectrometer for protein identification and quantification. Our results indicate that the label-free method provides the deepest proteome coverage. However, the quantification is not as efficient as in the labeling-based approaches, especially for low-abundant proteins. Metabolic labeling and isobaric chemical labeling have improved quantification accuracy, precision, and reproducibility. iTRAQ and TMT have similar performance in all aspects.

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Supporting Information Available:

Supplemental tables 1-3 and supplemental figures 1-2. This material is available free of charge via the Internet at http://pubs.acs.org

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Figure 1. Experiment design. Three *P. putida* cultures were grown in parallel, except that the culture 3 was metabolically labeled with ^{15}N. Proteins were extracted from cell cultures and digested into peptides which were measured using LC-MS/MS. In the label-free method, the culture 1 and 2 were prepared and measured separately. In metabolic labeling, the culture 1 and 3 were mixed at the beginning. In isobaric chemical labeling, peptides from the culture 1 and 2 were mixed after isobaric chemical labeling with TMT or iTRAQ.

Figure 2. Peptide quantification results at different reporter ion intensities of iTRAQ and TMT. Part A (iTRAQ) and B (TMT) show 2-dimensional histograms of peptide log_2ratio versus the associated log_2intensity for reporter ions. The color encodes the frequency of peptides at a given log_2ratio and log_2intensity. Then, the entire intensity range was split into eight bins. Median and median absolute deviation were calculated and plotted for each bin (Part C). As reporter ion intensity increased, quantification accuracy was improved. The value of MAD was independent of reporter ion intensity.

Figure 3. Distributions of quantified protein log_2ratios and peptide log_2ratios. Density plots were generated for log_2ratios quantified by each method at the protein level (A) and at the peptide level (B). iTRAQ and TMT produced narrower log_2ratios distributions than metabolic labeling and label-free at both the protein level and the peptide level, which indicates higher quantification precision.

Figure 4. Quantification reproducibility. Two-dimensional histograms were plotted to represent log_2ratios measured from the two technical replicates of each method (Part A: label-free (R^2 = 0.2); Part B: metabolic labeling (R^2 = 0.77); Part C: iTRAQ (R^2 = 0.87); D: TMT (R^2 = 0.87)). The color encodes the frequency of proteins quantified at log_2ratios in the two replicates. Quantification reproducibility was significantly improved in the labeling-based approaches.

Table 1. Protein identification results from label-free, metabolic labeling, iTRAQ, and TMT.

Table 2. Protein quantification results from label-free, metabolic labeling, iTRAQ, and TMT.

[a]: data is not available.

Supplemental Figure 1. Protein identification reproducibility. The Venn diagrams show the overlap of protein identifications between the duplicate runs (Part A: Label-Free; Part B: Metabolic Labeling; Part C: iTRAQ; and Part D: TMT). The red circle and the blue circle

represent proteins identified in run 1 and run 2, respectively. More than 70% of proteins were reproducibly identified between the duplicate runs.

Supplemental Figure 2. Reproducibility of spectral counting method. 2-dimensional histograms were constructed using $\log_2$ spectral counts of protein measured in the duplicate runs of culture 1 (A) and culture 2 (B). The color encodes protein frequency in the 2-dimensional histograms. Proteins with higher spectral counts have more similar spectral counts between the duplicate runs.

Supplemental Table 1. Protein IDs identified from each method.

[a]: locus ID of a protein;

[b]: the number of identified peptide matching to a protein;

[c]: the number of identified spectrum matching to a protein;

[d]: the percentage of a protein sequence that was identified;

[e]: the number of amino acid of a protein;

[f]: molecular weight of a protein;

[g]: isoelectric point of a protein;

[h]: annotation of a protein;

Supplemental Table 2. Protein IDs quantified from each method.

[a]: lower end of confidence interval of $\log_2$ratio of a protein;

[b]: upper end of confidence interval of $\log_2$ratio of a protein;

Supplemental Table 3. Peptides quantified from each method.

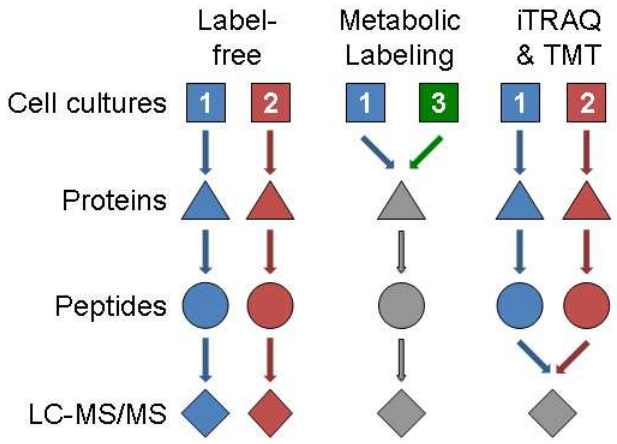

Label-
free
Metabolic
Labeling
iTRAQ
& TMT
Cell cultures
1
2
1
3
1
2
Proteins
Peptides
LC-MS/MS

Figure 1

	Label-Free				Metabolic Labeling		iTRAQ		TMT	
Run	Culture 1 Run 1	Culture 2 Run 1	Culture 1 Run 2	Culture 2 Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Spectrum count	58674	61440	43595	49389	52348	64972	29926	29328	35826	32897
Peptide count	12391	12727	11472	11184	9862	9618	7317	8248	6464	6795
Lys: Arg peptide ratio	0.64	0.66	0.68	0.69	0.67	0.57	0.68	0.76	0.66	0.70
Protein count	1687	1607	1598	1516	1447	1394	1202	1353	1239	1233
Average spectrum count per peptide	4.7	4.8	3.8	4.4	5.3	6.7	4.0	3.6	5.5	4.8
Average peptide count per protein	7.3	7.9	7.2	7.4	6.8	6.9	6.1	6.1	5.2	5.5
Average sequence coverage	24.6%	25.8%	23.6%	24.1%	22.3%	23.2%	19.9%	19.3%	16.5%	17.3%
Genome coverage	32.1%	30.6%	30.4%	28.9%	27.6%	26.6%	22.9%	25.8%	23.6%	23.5%

Table 1

	Label-free		Metabolic labeling		iTRAQ		TMT	
	Run 1	Run2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Median of protein log_2ratio	0.07	0.10	0	0	-0.02	0.00	0.00	0.00
Median of peptide log_2ratio	N/A	N/A	0.02	0.03	-0.13	-0.14	-0.07	-0.06
Median absolute deviation of protein log_2ratio	0.40	0.43	0.3	0.3	0.17	0.17	0.17	0.05
Median absolute deviation of peptide log_2ratio	N/A [a]	N/A	0.37	0.35	0.22	0.24	0.20	0.18
Percentage of proteins with log_2ratio within [-1,1]	87%	84%	94%	93%	99%	98%	99%	100%
Percentage of peptides with log_2ratio within [-1,1]	N/A	N/A	86%	86%	98%	98%	99%	99%
Number of quantified proteins	1174	1116	1327	1300	1185	1338	1231	1215
Number of quantified spectra	N/A	N/A	23331	24300	20919	21447	24818	23147

Table 2

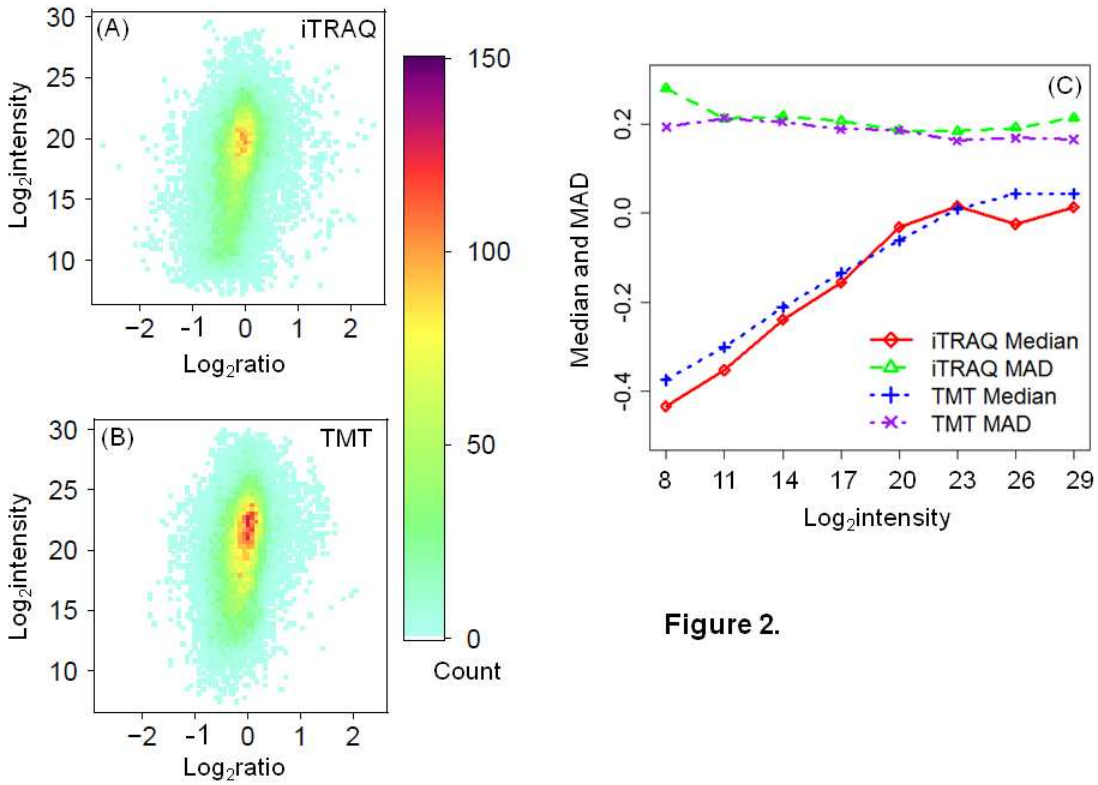

(A)
iTRAQ
(B)
TMT
Log2intensity
Log2ratio
Count
(C)
Median and MAD
Log2intensity
iTRAQ Median
iTRAQ MAD
TMT Median
TMT MAD

Figure 2.

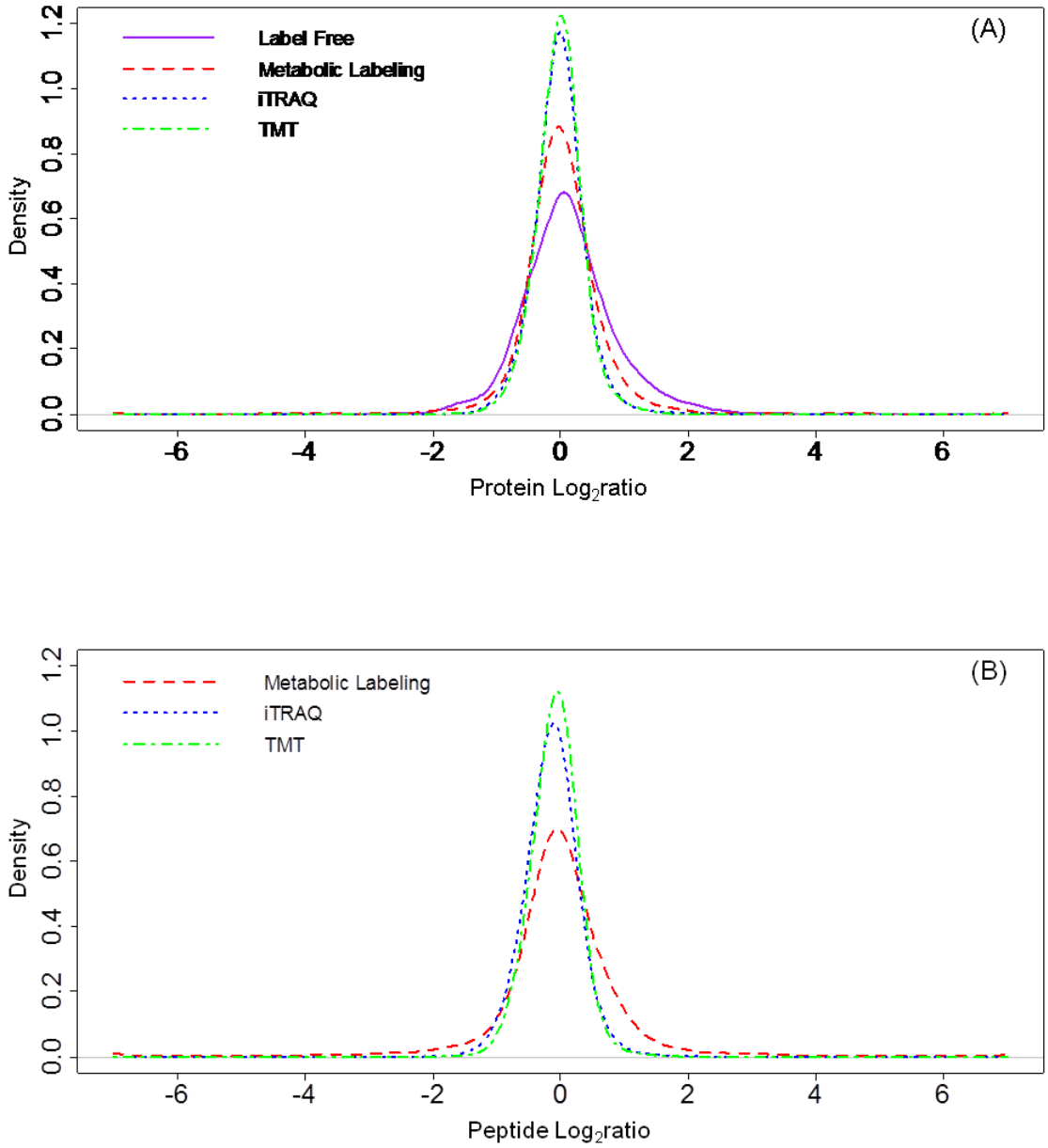

(A)
Label Free
Metabolic Labeling
iTRAQ
TMT
Density
0.0
0.2
0.4
0.6
0.8
1.0
1.2
-6
-4
-2
0
2
4
6
Protein Log2ratio
(B)
Metabolic Labeling
iTRAQ
TMT
Density
0.0
0.2
0.4
0.6
0.8
1.0
1.2
-6
-4
-2
0
2
4
6
Peptide Log2ratio

Figure 3

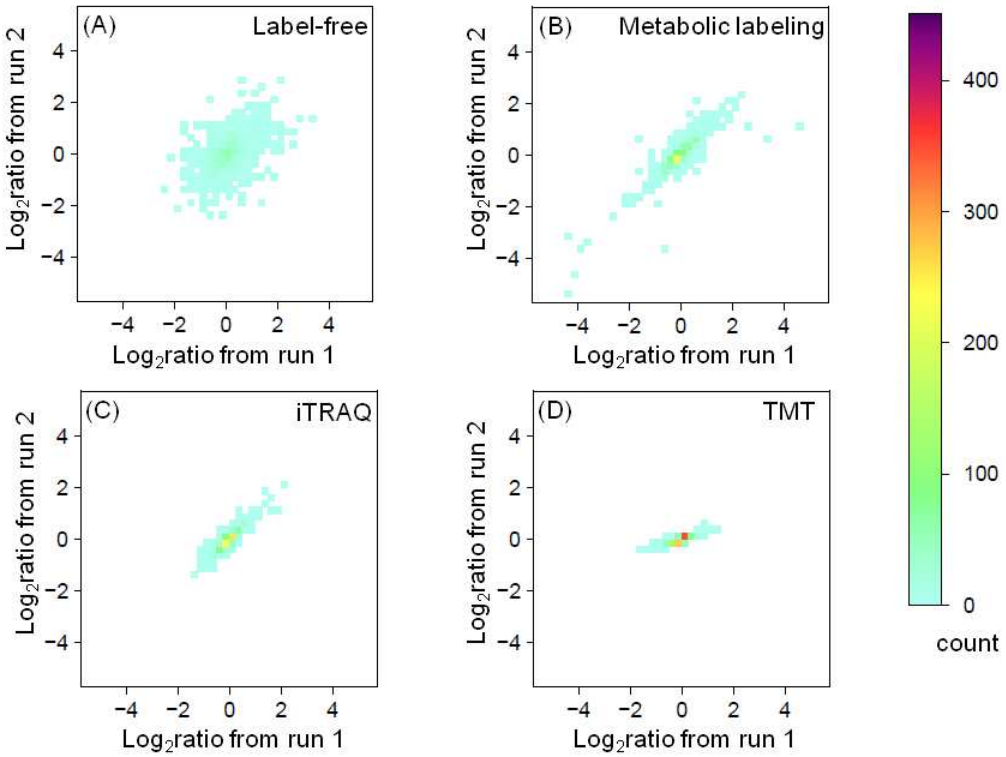
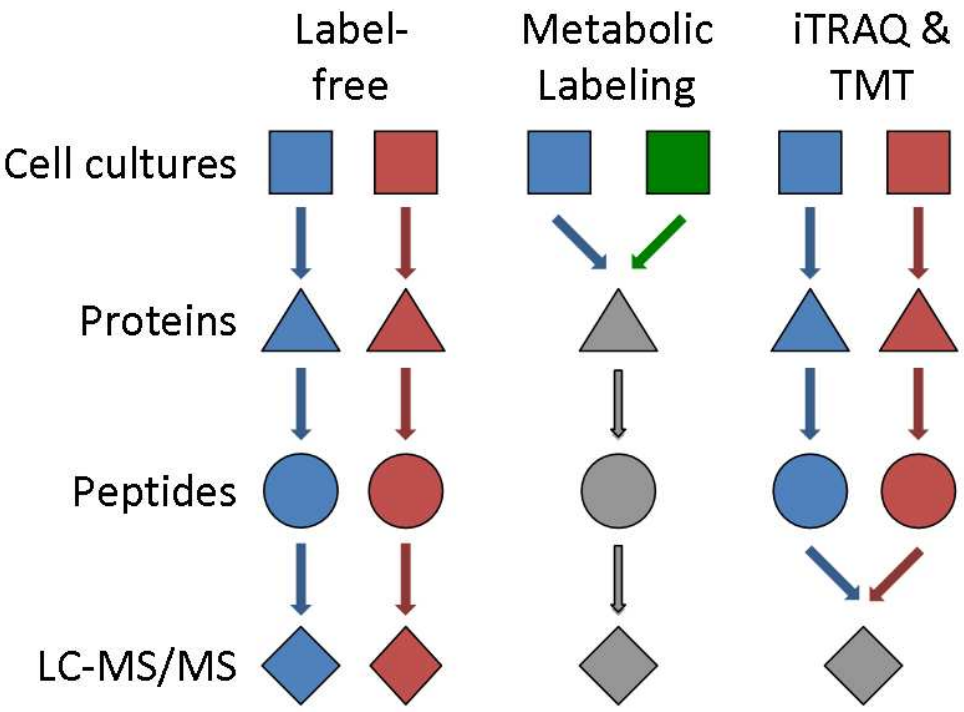

(A)
Label-free
(B)
Metabolic labeling
(C)
iTRAQ
(D)
TMT
Log2ratio from run 2
Log2ratio from run 1
−4
−2
0
2
4
400
300
200
100
0
count

Figure 4

Table of Contents (TOC) Graphic & Synopsis

Synopsis: Four quantitative proteomic methods, including label-free, metabolic labeling, and isobaric chemical labeling by iTRAQ or TMT, were compared using an LTQ Orbitrap Velos mass spectrometer for protein identification and quantification. The label-free method generated the deepest proteome coverage. Metabolic labeling and isobaric chemical labeling had significantly improved quantification accuracy, precision, and reproducibility. Each method provides unique capabilities for quantitative proteomics.