Comparative Evaluation of Two Isobaric Labeling Tags, DiART and iTRAQ

Zhen Chen,§ Quanhui Wang,‡ Liang Lin,‡ Qi Tang,‡ James L. Edwards,‡§ Shuwei Li,‡§ and Siqi Liu†,‡,§

† Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, 101318, China
‡ BGI-Shenzhen, Shenzhen, 518083, China
§ Graduate University of the Chinese Academy of Sciences, Beijing, 100049, China

ABSTRACT: Isobaric tags have broad applications in both basic and translational research, as demonstrated by the widely used isobaric tag for relative and absolute quantitation (iTRAQ). Recent results from large-scale quantitative proteomics projects, however, indicate that protein quantification by iTRAQ is often biased in complex biological samples. Here, we report the application of another isobaric tag, deuterium isobaric amine reactive tag (DiART), for quantifying the proteome of Thermanaerobacter tengcongensis (T. tengcongensis), a thermophilic bacterium first discovered in China. We compared the performance of DiART with iTRAQ from several different aspects, including their fragmentation mechanisms, the number of identified proteins, and the accuracy of quantification. Our results revealed that, as compared with iTRAQ, DiART yielded significantly stronger reporter ions, which did not reduce the number of identifiable peptides, but improved the signal-to-noise ratio (S/N) for quantification. Remarkably, we found that, under identical chromatography and mass spectrometry (MS) conditions, DiART exhibited less reporter ions ratio compression than iTRAQ, probably due to more reporter ions with higher intensities produced by DiART labeling. Taken together, we demonstrate that DiART is a valuable alternative of iTRAQ with enhanced performance for quantitative proteomics.

© 2012 American Chemical Society

Published: February 29, 2012
Accepted: February 29, 2012
Received: December 26, 2011

Cell mass spectrometry (MS) is one of few methods with such capability and has therefore become a widely utilized tool for profiling proteomes. Unfortunately, MS itself is not a good tool for quantitative measurement because its signal intensity is prone to the influence of many parameters, such as physical–chemical properties of analytes and matrix effects. To overcome this problem, stable isotope labeling approaches have been developed to allow multiple biological samples to be analyzed under the same chromatography and MS conditions. The unpredictable factors in quantitative proteomics thus are removed, and more accurate quantitative information could be achieved. These methods, on the basis of how proteins or peptides are labeled and quantified, can be grouped into two large categories, mass-shift tags and isobaric tags. A peptide labeled with mass-shift tags shows distinct peaks with a fixed mass difference in an MS spectrum, whose intensity reflects the abundance of this peptide in original samples. Some popular techniques using mass-shift tags, such as stable isotope labeling with amino acids in cell culture (SILAC) and isotope-coded affinity tags (ICAT), have been generally employed in quantitative proteomics. On the other hand, approaches with isobaric tags have emerged in recent years and played a more important role to quantify proteomes. Isobaric tags do not introduce mass difference into labeled peptides because they are isotope-coded molecules with the same chemical structure and molecular weight. Their structure consists of a reporter and a balancer containing different isotopes positioned in a way that the different masses of the reporters in a set of reagents are compensated by those of the balancers. In addition, the
Chemical bond linking the reporter and the balancer is breakable under collision induced dissociation (CID) conditions in MS. Taken together, a peptide differentially labeled by a set of isobar tags, albeit displaying a single peak on an MS spectrum, can yield a series of low-mass reporter ions for quantification upon tandem mass spectrometry (MS/MS) fragmentation. Currently, there are two popular isobaric reagents available in market, tandem mass tag (TMT) and isobaric tag for relative and absolute quantitation (iTRAQ). Up to eight samples can be quantified concurrently by these reagents, thereby improving both the throughput and reproducibility of MS analysis.

Most isotope labeling reagents for large-scale proteomics experiments do not use deuterium (2H) as one of the heavy isotopes because 2H can introduce a detrimental chromatographic shift in reverse phase liquid chromatograph (RP-LC) and compromise the accuracy of quantification. Therefore, TMT and iTRAQ tags only contain 13C, 15N, and 18O as coding isotopes, partly contributing to the high cost associated with their applications. Interestingly, the 1H-associated isotope effect in RP-LC has been recently found to be negligible when H atoms are placed close to hydrophilic groups like tertiary amines. On the basis of this observation, we developed a set of isobaric reagents, deuterium isobaric amine reactive tag (DiART), and showed that they can quantify both proteins and small molecule metabolites. In contrast to TMT and iTRAQ, the synthesis of DiART is much easier, making it more cost-effective for applications that require labeling a large quantity of samples. In addition, DiART reagents show high chemical and isotope purity, also thanks to their short synthetic routes.

We previously tested the performance of DiART reagents on a simplified system: tryptic peptides digested from a mixture of three proteins. To make DiART useful for the proteomic analysis of biological samples as well as for the proteomics community, here, we demonstrate the application of DiART on evaluating temperature-dependent protein expression in *Thermoanaerobacter tengcongensis* (T. tengcongensis). This thermophilic bacterium, which was first isolated from China and comprehensively studied at its genomics and proteomics, is an ideal model in exploring the molecular mechanisms of thermophilic survival. Wang applied two-dimensional electrophoresis (2-DE) to separate the bacterial proteins and used peptide mass fingerprinting to identify the temperature-dependent proteins. The abundance of many *T. tengcongensis* proteins were found to be changed when cultured at different temperatures. The investigation enlightens our view on the thermal tolerant proteins that facilitate the survival of thermophilic bacteria; however, due to technique limit, it still leaves a lot of room for finding more temperature-dependent proteins. As a result, our experiments with DiART labeling would not only complement our previous knowledge on the life cycle of *T. tengcongensis* but also establish a more powerful platform for large scale quantitative proteomics in biological samples. Meanwhile, we ran a side-by-side comparison of DiART and iTRAQ tags to evaluate their performances. We achieved satisfactory quantification data, indicating that DiART is a valuable stable isotope labeling method with enhanced performance in quantitative proteomics. Moreover, DiART offers significantly stronger reporter ions than that of iTRAQ, which is likely to detect more quantitative signals.

### Materials and Methods

**Materials.** All solvents (HPLC-grade) were purchased from J. T. Baker (Philipsburg, NJ, USA). Sequence-grade trypsin was obtained from Promega (Madison, WI, USA). The chemicals for culture media were from Oxoid (Basingstoke, Hampshire, UK). The DiART reagents were synthesized and prepared as previously reported. The iTRAQ reagents Multiplex kit was purchased from AB Sciex (Foster City, CA, USA). Other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

**T. tengcongensis Growth.** *T. tengcongensis* strain MB4 was cultured in the media as previously described under four different temperatures, 55, 65, 75, and 80 °C, respectively. The bacterial cells were harvested at the middle stage of log phase when OD600 approximately reached 0.6. The cell pellets were collected after centrifugation at 4000g at 4 °C, washed twice with an isotonic buffer (50 mM Tris-HCl, pH = 7.8), and stored at −80 °C until use.

**Protein Extraction and Digestion.** The cell pellets were suspended in the lysis buffer (8 M urea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol (DTT), and 40 mM Tris-HCl, pH = 8.0) and sonicated in ice. The proteins were reduced with 10 mM DTT at 56 °C and then alkylated by 55 mM iodoacetamide in the dark at room temperature. The treated proteins were reprecipitated in 80% acetone at −20 °C overnight and redissolved in 0.8 M urea and 500 mM tetraethylammonium bicarbonate (TEAB), pH 8.5. After centrifuging at 12 000g at 4 °C, an aliquot of the supernatant was taken for determination of protein concentration by the Bradford method. The proteins in TEAB were digested by trypsin at 37 °C, and the solvent was removed by Speedvac.

**Isobaric Tag Labeling.** Basically, the 6-plex DiART labeling was similar as previously reported with slight modifications. The DiART reagents (0.8 mg) were dissolved in 48 μL of anhydrous acetonitrile. The labeling reaction mixture contained 45 μL of the DiART reagent and 20 μL of the peptide solution (100 μg of peptide in 500 mM TEAB). After the labeling reaction was kept at room temperature for 2 h, the treated proteins were reprecipitated in 80% acetone at −20 °C overnight and redissolved in 0.8 M urea and 500 mM tetraethylammonium bicarbonate (TEAB), pH 8.5. After centrifuging at 12 000g at 4 °C, an aliquot of the supernatant was taken for determination of protein concentration by the Bradford method. The proteins in TEAB were digested by trypsin at 37 °C, and the solvent was removed by Speedvac. The labeled peptides were ready for LC-MS/MS.

**Identification of the Labeled Peptides by LC-MS/MS.** The labeled peptides were dissolved in 0.1% formic acid solution and delivered into a RP nano-HPLC column (75 μm inner diameter × 150 mm length filled with 5 μm Hypersil C18 resin) mounted in a Prominence Nano HPLC system ( Shimadzu, Nakagyo-ku, Kyoto, Japan). The peptides were eluted with acetonitrile gradient from 5% to 40% for 95 min at 400 nL/min. The eluent from nano-HPLC directly entered a RP nano-HPLC column (75 × 150 mm length filled with 5 μm Hypersil C18 resin) mounted in a Prominence Nano HPLC system ( Shimadzu, Nakagyo-ku, Kyoto, Japan). The peptides were eluted with acetonitrile gradient from 5% to 40% for 95 min at 400 nL/min. The eluent from nano-HPLC directly entered a LTQ-Orbitrap Velos MS (Thermo Fisher Scientific, Waltham, MA, USA). The mass signals were acquired in positive ion mode with data-dependent MS/MS acquisition. For scanning of the labeled peptides, the resolution was set at 60 000 and the m/z window was set from 350 to 1800. For fragmentation of the labeled peptides, concomitant fragmentation of CID and higher energy C-trap dissociation (HCD) mode was used.
conducted and the resolution in HCD mode was set at 7500. Variable normalized collision energies (NCEs) were tested for optimizing the fragmentation of either DiART or iTRAQ labeled peptides. Finally, a proper fragmentation parameter set was established for labeled peptides, in which the NCE was 35% in CID mode and the NCE was 45% in HCD mode.

To evaluate the correlation between confidences and fold changes of the identified proteins, two-dimensional LC for peptide separation was used. At the first dimension, peptides were fractionated by a gradient of acetonitrile in 20 mM ammonium formate (pH = 10) from 10% to 40% in 60 min, and 12 fractions were collected. At the second dimension, the chromatographic conditions were the same as described above.

Data Analysis. The raw data was converted into MGF format with the software MM File conversion, version 3.9 (http://www.massmatrix.net/mm-cgi/downloads.py). A program to combine the CID and HCD MS/MS spectra for the same precursor into a single spectrum was written in house using Perl script. The combined MS/MS spectra were analyzed with Mascot 2.3 (Matrix Science, Boston, MA, USA) for protein identification as well as protein quantification. For protein identification, the sequence database of *T. tengcongensis* was downloaded from NCBI. Carbamidomethylation on cysteine was set as a fixed modification, and oxidation on methionine was set as a variable modification. The peptide mass tolerance was 10 ppm, and the fragment ion tolerance was either 0.5 Da for CID in LTQ or 0.02 Da for HCD in Orbitrap. An automatic decoy database search was also performed. For protein quantification, those proteins with at least two unique peptides (FDR < 0.05) were qualified for further quantification data analysis. The protein fold changes were reported as the median ratio of all significantly matched peptides.

### RESULTS AND DISCUSSION

Quantification of isobaric labeled peptides is achieved after MS/MS fragmentation, in which low-mass reporter ions are used for measuring the relative intensities of differentially labeled peptides and other large fragments are used for peptides identification. To improve the signal-to-noise ratio (S/N) of quantification, it is desirable to develop tags capable of producing high-intensity reporter ions, but strong reporter ions can also suppress the generation of other large fragments, which are needed for high-confidence peptide sequence identification. Therefore, both factors should be considered when designing a new isobaric tag. Ideally, the collision energy required for breaking reporter ions from a labeled parent peptide should be slightly lower than that for a standard peptide bond. Peptides labeled by tags meeting this criterion would provide both strong reporter ions for accurate quantification and enough large fragments for reliable peptide assignment. However, because the behavior of peptides with different sequences is hard to predict during MS/MS fragmentation, it is difficult to develop a general structure satisfying all peptides with diverse sequences. To date, only a few structures have been applied for isobaric labeling. TMT and iTRAQ are based on *N*,*N*-dialkylated glycine, while DiART and similar DiLeu are derived from *N*,*N*-dimethyl leucine.

![Figure 1. Comparison of reporter ion intensity generated from DiART and iTRAQ tags.](image)
We previously reported that DiART reagents are easier to synthesize than TMT and iTRAQ.\textsuperscript{11} It would also be important to examine the performance of DiART for large scale quantitative proteomics analysis in order to evaluate whether DiART can provide a cost-effective replacement of TMT and iTRAQ. For this purpose, proteins extracted from \textit{T. tengcongensis} cells grown at different temperatures (55, 65, 75, and 80 °C) were digested with trypsin and reacted with four DiART tags (114-117), respectively. Differentially labeled peptides were mixed and analyzed by a hybrid CID/HCD data-dependent acquisition method, in which each parent peptide is fragmented twice by CID and HCD.\textsuperscript{15} In an ion-trap based instrument like LTQ-Orbitrap, HCD is required for analyzing isobaric labeled samples because low-mass reporter ions can only be detected in HCD spectra. CID, on the other hand, can provide better sequence coverage. To compare DiART with more popular iTRAQ, the same \textit{T. tengcongensis} samples were also labeled by four iTRAQ reagents (114-117) and analyzed with the same hybrid CID/HCD method.

**Labeling Efficiency.** To determine protein quantification accurately with isotope tags, labeling efficiency needs to reach 100%. DiART reagents contain an N-hydroxysuccinimide ester (NHS), which is an amine reactive group with required specificity and efficiency. TMT and iTRAQ tags also use the same chemistry, yet whether DiART reagents can completely label both N-terminus amine and ε-amine of lysine on thousands of tryptic peptides with different sequences depends on many factors, such as reaction conditions, the purity of reagents, and the molar ratio of reagents over amine on peptides. To test the labeling efficiency of DiART reagents, we analyzed the \textit{T. tengcongensis} peptides labeled by DiART on a LC coupled LTQ-Orbitrap Velos and then carried out database searching on a Mascot server by setting DiART tags as a variable modification on either N-terminus amine or lysine. This is different from the standard database searching algorithm, in which DiART tag is set as a fixed modification. With this new parameter set, both labeled and unlabeled peptides can be recognized and used to calculate labeling efficiency, which is defined as the percent of labeled peptides among all identified peptides. We found that the labeling efficiency of the two approaches were quite comparable: DiART (98.1%) versus iTRAQ (99.6%). Because of the high labeling efficiency and the negligible difference of labeling efficiency between the two methods, we concluded that both labeling reactions were completed, and the two labeling methods were comparable in quantitative evaluation in parallel.

**Reporter Ion Intensity.** Our results indicated the reporter ions generated from DiART are significantly higher than those from iTRAQ. For example, the reporter ions from a DiART-labeled peptide (LAGGVAVIQVGAATETELK) are the most dominating peaks on its HCD spectrum (Figure 1A). In contrast, the reporter ions produced from the same peptide labeled by iTRAQ tags are only at 10% intensity of the highest peak. We believe this observation results from the different structures of DiART and iTRAQ tags. The MS/MS fragmentation of DiART tags can yield a secondary carbocation intermediate, which is more stable than the primary carbocation intermediate generated by iTRAQ tags (Figure 1B). Both intermediates then rearrange to more energy-favored quaternary amines as the final reporter ions. As a result, the chemical bond linking the reporter and the balancer in a DiART-label peptide is easier to fragment than that in an iTRAQ-labeled counterpart, regardless of the peptide sequence. In order to confirm this conclusion, we compared the intensity of one reporter ion (116) extracted from 8147 spectra of a DiART-labeled sample and 5878 spectra of an iTRAQ-labeled counterpart. The DiART-produced reporter ions are much higher than those from iTRAQ (Figure 1C). This would increase the number of quantifiable peptides, especially those peptides at low abundance. In addition, we found that the accuracy of quantifying DiART-labeled peptides is dependent on the intensity of reporter ions. Figure 1D shows the ratio of two reporter ions generated from DiART-labeled peptides in a technical replicate experiment at logarithm scale (\textit{log}_{10}(116/117) at y-axis) versus their average ion intensity ((116 + 117)/2) at x-axis). In this experiment, the same sample was differentially labeled by two DiART tags (116 and 117) and mixed at equal amount for analysis, so the ratio of all reporter ions should theoretically be close to 1:1 under ideal conditions. However, the actual ratio deviates from 1:1 significantly when the parent ion intensity is lower than 10 000 counts. This result is consistent with previous findings by others using iTRAQ for peptide quantification,\textsuperscript{16} suggesting it is a general phenomenon that stronger reporter ions can provide more reliable...
quantitative results. Therefore, quantifying peptides with DIART would be more accurate for low-abundance peptides than using iTRAQ, thanks to high-intensity reporter ions generated by DIART tags.

**Peptide Identification.** The easy fragmentation of DIART tags might be a double-edged sword for peptide identification because increased reporter ion intensity is often associated with the decreased signal of other large peptide fragments. It is critical to examine whether this would reduce the number of identifiable peptides. Previously, we only used CID data for peptide identification and reserved HCD data for peptide quantification.\(^9\) We recently found that HCD can complement CID for peptide identification because these two fragmentation methods sometime recognize different set of peptides. We therefore developed a modified protocol for analyzing data generated from hybrid CID/HCD experiments. As shown in Figure 2A, CID and HCD data are first extracted separately from a raw data set. The low-mass ion peaks (\(m/z < 200\)) and precursor related peaks due to neutral loss (peaks within the range of M-148 to M-142, M is the molecular weight of each precursor calculated by multiplying its \(m/z\) and \(z\)), as well as their associated doubly or triply charged species, are removed from CID spectra as this treatment can improve the score of identified peptides.\(^{13d}\) The reporter ions in each HCD spectrum are then normalized to the strongest reporter in this spectrum, extracted, and inserted into each respective CID spectrum. This step allows CID spectra to be used for peptide quantification as well. Finally, the processed CID spectra are merged with HCD spectra to form a single data file for database searching. This procedure is accomplished with a home-written Perl script. Compared to our previous approach for data analysis, this new protocol can increase the number of both identifiable and quantifiable peptides (data not shown).

Using this data analysis method, we examined the impact of NCE on the fragmentation and identification of DIART-labeled peptides (Figure 2B). We first tested the effect of CID energy, in which the NCE of HCD was fixed at 45% and the NCE of CID was varied at 30%, 35%, and 40%. The total peptides identified under these conditions were 3071, 3054, and 2998, while the numbers of unique peptides were 1519, 1520, and 1499, respectively. At protein level, 453, 450, and 470 proteins were identified. These results suggest that the fragmentation of DIART-labeled peptides is not significantly affected by the CID energy in LTQ-Orbitrap and the default value (35% NCE) recommended by the vendor can be used in routine experiments. Next, we tested the effect of HCD energy on peptide identification by keeping CID at 35% NCE and setting HCD at 40% NCE. This experiment allowed us to identify 2973 total peptides, 1445 unique peptides, and 420 proteins, which were slightly fewer than what we obtained from experiments with HCD at 45% NCE. Therefore, we chose CID at 35% NCE and HCD at 45% NCE as the standard condition to analyze DIART-labeled samples.

As a comparison, an iTRAQ-labeled *T. tengcongensis* sample was also analyzed at 35% NCE for CID and 45% NCE for HCD. We found that higher collision energy in HCD (60% NCE) can only slightly increase the reporter ion intensity (up to 30%) of iTRAQ-labeled peptides, while significantly

Figure 3. (A) Venn diagram of quantified proteins by DIART and iTRAQ. An iTRAQ labeled sample and a DIART labeled sample were analyzed under identical conditions. Among 343 proteins detected by both methods, 15 proteins are quantified by neither tag. 309 (dashed-line circle) and 297 (solid-line circle) proteins are quantified by DiART and iTRAQ, respectively, with 278 proteins quantified by both tags. (B) Heat map corresponding to the relative abundance (log2(ratios) between two reporter ions) of 278 proteins quantified by both DiART and iTRAQ, Lane 1: 115/114; Lane 2: 116/114; Lane 3: 117/114. The right panel with color gradient represents the changes of protein abundances (log2(ratios)) from down-regulated (green) to up-regulated (red). (C) Volcano plots showing the confidence and fold change of quantified proteins from *T. tengcongensis* grown at two temperatures (55 °C, labeled by tag 114; 75 °C, labeled by tag 116). Spots inside gray shadow boxes are highly confident (\(p\)-value < 0.05) and differentially expressed (fold change > 1.5) proteins. Left plot shows the overlay of the DIART-labeled sample (dark yellow squares) and the iTRAQ-labeled sample (green circles) without prefractionation. Right plot shows the overlay of the iTRAQ-labeled sample without prefractionation (green circles) and the same iTRAQ-labeled sample with prefractionation (dark blue triangles). The fold change and \(p\)-value of one protein (gi20517337) determined under these three conditions (DiART-labeling, without prefractionation, squares; iTRAQ-labeling, without prefractionation, circles; iTRAQ-labeling, with prefractionation, triangles) are highlighted in red. (D) Venn diagram of the differentially expressed proteins by DIART and iTRAQ (fold change > 1.5 and \(p\)-value < 0.05). 114 proteins were determined to be differentially expressed by DIART (dashed-line circle), while 56 proteins were from iTRAQ (solid-line circle). 41 of them were shared by both methods.

dx.doi.org/10.1021/ac203467q
Table 1. Fold Changes of Some Differentially Expressed Proteins Identified from Three Distinct Methods, 2-DE, iTRAQ, and DiART

<table>
<thead>
<tr>
<th>energy production and conversion</th>
<th>gene ID</th>
<th>product name</th>
<th>gene locus</th>
<th>2-DE loci</th>
<th>iTRAQ labeling</th>
<th>DiART labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTE0273</td>
<td>galactose-l-phosphate uridylytransferase ruberythrin</td>
<td>GadT</td>
<td></td>
<td></td>
<td>1.051</td>
<td>0.607</td>
</tr>
<tr>
<td>TTE0487</td>
<td></td>
<td></td>
<td>-</td>
<td>decreased</td>
<td>0.371</td>
<td>0.287</td>
</tr>
<tr>
<td>TTE0637</td>
<td>FO1 ATP synthase subunit beta</td>
<td>AtpD</td>
<td></td>
<td></td>
<td>0.674</td>
<td>0.609</td>
</tr>
<tr>
<td>TTE0688</td>
<td>thiamine pyrophosphate-dependent dehydrogenase, El component alpha subunit</td>
<td>AcoA2</td>
<td></td>
<td></td>
<td>0.971</td>
<td>0.434</td>
</tr>
<tr>
<td>TTE0689</td>
<td>thiamine pyrophosphate-dependent dehydrogenase, El component beta subunit</td>
<td>AcoB2</td>
<td></td>
<td></td>
<td>0.467</td>
<td>0.335</td>
</tr>
<tr>
<td>TTE0690</td>
<td>dihydrolipoamide dehydrogenase</td>
<td>AceF2</td>
<td>55</td>
<td></td>
<td>1.746</td>
<td>0.406</td>
</tr>
<tr>
<td>TTE0890</td>
<td>NADH/ubiquinone oxidoreductase 24 kD subunit</td>
<td>NuoE</td>
<td>55</td>
<td></td>
<td>0.848</td>
<td>0.425</td>
</tr>
<tr>
<td>TTE0892</td>
<td>ferredoxin</td>
<td></td>
<td>55</td>
<td></td>
<td>0.512</td>
<td>0.418</td>
</tr>
<tr>
<td>TTE0893</td>
<td>NADH/ubiquinone oxidoreductase, NADH-binding (51 kD) subunit</td>
<td>NuoF</td>
<td>0.534</td>
<td>0.424</td>
<td>0.416</td>
<td>0.618</td>
</tr>
<tr>
<td>TTE1000</td>
<td>uncharacterized flavoproteins</td>
<td></td>
<td></td>
<td></td>
<td>0.601</td>
<td>0.651</td>
</tr>
<tr>
<td>TTE1708</td>
<td>carbon monoxide dehydrogenase subunit CooS</td>
<td></td>
<td></td>
<td></td>
<td>0.654</td>
<td>0.383</td>
</tr>
<tr>
<td>TTE2002</td>
<td>glycerol kinase</td>
<td>G1pK</td>
<td></td>
<td></td>
<td>0.741</td>
<td>0.665</td>
</tr>
<tr>
<td>TTE2131</td>
<td>NADH/flavin oxidoreductase</td>
<td>NemA3</td>
<td></td>
<td></td>
<td>0.862</td>
<td>0.614</td>
</tr>
<tr>
<td>TTE2198</td>
<td>2-ketoisovalerate ferredoxin reductase</td>
<td>PorA6</td>
<td></td>
<td></td>
<td>0.987</td>
<td>0.708</td>
</tr>
<tr>
<td>TTE2486</td>
<td>NADH dehydrogenase, FAD-containing subunit</td>
<td>Ndh</td>
<td></td>
<td></td>
<td>0.743</td>
<td>0.587</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>chaperones</th>
<th>gene ID</th>
<th>product name</th>
<th>gene locus</th>
<th>2-DE loci</th>
<th>iTRAQ labeling</th>
<th>DiART labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTE0579</td>
<td>co-chaperonin GroES (HSP10)</td>
<td>groES</td>
<td>increased</td>
<td></td>
<td>1.455</td>
<td>2.633</td>
</tr>
<tr>
<td>TTE0580</td>
<td>chaperonin GroEL</td>
<td>groEL</td>
<td>increased</td>
<td></td>
<td>1.057</td>
<td>1.599</td>
</tr>
<tr>
<td>TTE2327</td>
<td>ATPase with chaperone activity, ATP-binding subunit</td>
<td>CtpA</td>
<td></td>
<td></td>
<td>1.134</td>
<td>1.268</td>
</tr>
<tr>
<td>TTE2578</td>
<td>molecular chaperone (small heat shock protein)</td>
<td>IlbP</td>
<td></td>
<td></td>
<td>1.302</td>
<td>1.706</td>
</tr>
<tr>
<td>TTE2674</td>
<td>co-chaperonin GroES (HSP10)</td>
<td>GroS2</td>
<td></td>
<td></td>
<td>1.21</td>
<td>1.413</td>
</tr>
<tr>
<td>TTE0955</td>
<td>molecular chaperone</td>
<td>DnaK</td>
<td></td>
<td></td>
<td>1.212</td>
<td>1.996</td>
</tr>
</tbody>
</table>

“These proteins belong to two groups known to respond to thermal stress. Blank in a column means the protein was not identified or not quantified by this method. 55 in the 2-DE column indicate these proteins were only detected at 55 °C and not detected at higher temperatures. Ratios in other columns (i.e., 65/55, 75/55, 80/55) are the fold changes of this protein under two temperatures. Reducing the number of identified peptides, so we did not further study the effect of HCD energy on iTRAQ-labeled samples. Since it was reported that iTRAQ-labeled peptides can be analyzed by HCD alone,17 we also ran a HCD-only acquisition experiment at 45% NCE with the same iTRAQ-labeled sample. The CID/HCD analysis of iTRAQ-labeled sample led to the identification of 3133 total peptides, 1502 unique peptides, and 462 proteins, indicating DiART and iTRAQ have comparable performance for large-scale quantitative proteomics. Interestingly, the HCD-only experiment gave rise to 3294 total peptides, 1552 unique peptides, and 472 proteins, which were slightly better than the hybrid CID/HCD experiments of both DiART and iTRAQ-labeled samples. This discrepancy may be caused by the fact that CID/HCD approaches require slightly longer time for analyzing each precursor ion than the HCD only method. This would reduce the total number of peaks that are fragmented in a typical experiment and yield less MS/MS spectra available for peptide identification. Unlike iTRAQ, we found that DiART-labeled samples cannot be analyzed by the HCD-only approach due to easy fragmentation of its reporter ions. However, this small disadvantage of DiART in peptide identification can be easily made up by its significant advantage on peptide quantification.

**Peptide and Protein Quantification.** In typical large scale proteomics research, not all of the identified peptides and proteins are quantified because some labeled peptides do not show the reporter ions when fragmented. This is a less serious problem for DiART than iTRAQ since the fragmentation of DiART is easier than iTRAQ. When we compared the DiART- and the iTRAQ-labeled T. tengcongensis peptides acquired at the same conditions (CID at 35% NCE and HCD at 45% NCE), a total of 343 proteins were identified by both methods (Figure 3A). Among them, 15 proteins were quantified by neither tag. Nineteen proteins were quantified by iTRAQ only, and 31 proteins were quantified by DiART only. The remaining 278 proteins were quantified by both tags. We then plotted a heat map of these 278 proteins to examine whether iTRAQ and DiART can provide consistent quantification results (Figure 3B). On this heat map, the ratios among reporter ions (115/114, 116/114, and 117/114) reflect the amount of proteins in bacterial cells grown at different temperatures. Most proteins showed the same tendency of changes in response to culture temperatures, regardless of which labeling was used. However, the fold change of many proteins in the DiART labeled peptides was higher than that of iTRAQ labeled counterparts. To figure out what caused this disagreement, we performed a student’s t test to evaluate the protein expression profile of the DiART and iTRAQ labeled samples. Volcano plots were made to examine the relationship between the confidence (y-axis) and the fold change of each quantified protein (x-axis) (Figure
Those spots inside the gray shadow boxes represent proteins with high confidence (p-value < 0.05) and differential expression (fold change > 1.5). Using this criterion, we identified 56 and 114 proteins in this category from the iTRAQ- and the DiART-labeled samples, respectively, while 41 proteins were shared by both methods (Figure 3D). The number of differential proteins derived from the DiART labeling is 2-fold over that from the iTRAQ labeling. This discrepancy may be explained by the underestimation effect, a known problem of quantification methods based on isobaric tags. During analysis of a complex biological sample, a MS window for a particular parent ion can often select both the target and some contaminant ions with similar m/z values, which yield the same reporter ions upon MS/MS. This mixed MS/MS background can suppress potentially large intensity differences. Peptides producing low intensity reporter ions, either because of low abundance parent ions or because of inefficient fragmentation, are more susceptible to this level-off effect than those with high intensity reporter ions. Since DiART is easier to fragment and generates stronger reporters than iTRAQ, the quantification of DiART-labeled samples might be less influenced by this level-off problem and more differential proteins could be observed. To test this hypothesis, we prefractionated the iTRAQ-labeled sample with an alkaline RP-LC separation before the regular acidic RP-LC coupled MS/MS analysis. This procedure can reduce sample complexity in each LC-MS/MS run, which not only increases the number of identifiable proteins but also alleviates underestimation effect because fewer precursor ions are contaminated by other ions in a given MS window. Indeed, a total of 1391 proteins were identified, including 1373 proteins with quantitative tags. Many proteins that were not detected as significant changes without the prefractionation treatment were found as the temperature-dependent ones with this prefractionation step, indicating underestimation effect is indeed relieved by reducing sample complexity. One of such examples is protein gi20517337, a translation elongation and release factor. This protein was found to have a fold change of 1.35 (p-value = 0.022) without prefractionation, which is below the significance threshold (fold change > 1.5) but displayed a fold change of 2.5 (p-value = 0.016) after prefractionation (Figure 3C, right plot). When the DiART sample was analyzed without prefractionation, this protein was determined to have a fold change of 2.0 (p-value = 5.5 × 10^{-5}), well above the significance threshold and in alignment with the fold change of 2.5 observed in the prefractionation sample, suggesting DiART-labeled samples are less susceptible to this adverse effect than their iTRAQ-labeled counterparts (Figure 3C, left plot) under the same analytical conditions.

**Biological Significance.** We determined 56 and 114 temperature-dependent differential proteins from iTRAQ- and DiART-labeled samples, respectively, on the basis of the stringent criteria with fold change > 1.5 and p-value (t test) < 0.05. Although the number of differential proteins derived from the DiART labeling is obviously more than that from the iTRAQ labeling, the trends of abundance changes for *T. tengcongensis* proteins in response to elevated temperature were quite comparable in the two data sets.

According to the previous report on the temperature-dependent proteins of *T. tengcongensis*, Wang adopted the 2-DE technique and claimed 14 unique proteins displaying consecutive abundance changes responding to increased growth temperature. Of the temperature-dependent proteins, two functional groups, including four down-regulated proteins in the energy production group and two up-regulated proteins in the chaperon group, may have important biological significance, because energy reservation and denaturation protection are critical for bacteria survival at high temperature. The quantitative proteomic data achieved from DiART or iTRAQ described in Table 1 offers additional evidence to support the previous conclusion. Importantly, with improvement in accuracy as well as sensitivity in quantitative techniques, the results obtained upon DiART and iTRAQ, provide more information that helps elucidate thermophilic mechanisms. For instance, four proteins related with energy production were identified in down-regulation mode by 2-DE, whereas 15 proteins in the same functional group, including all the four proteins detected by 2-DE, were recognized as the down-regulated ones in response to increasing temperature in this study. Of these 15 proteins, 14 proteins were identified by the DiART labeling, while 7 proteins were also discovered by iTRAQ labeling. Furthermore, in addition to two up-regulated chaperon proteins in response to elevated temperature identified by the 2-DE method, four more chaperon proteins with dramatically boosted abundance were identified by the labeling approaches. These results suggest all the components of chaperon complexes in *T. tengcongensis* cells may perform similar functions in response to elevated temperature. Therefore, our observation delivers a clear message that the labeling approach upon quantification of mass signals possesses an advantage over the 2-DE approach upon quantification of image signals for exploring the differential proteomes, even for soluble proteins within the resolvable mass range of 2-DE.

### CONCLUSIONS

Isobaric based approaches have provided a robust tool for both basic and translational research as they offer a sensitive, high-throughput, and accurate way for large scale protein quantification. TMT and iTRAQ, two popular isobaric reagents, have been widely used for a broad range of applications, including the identification of posttranslational modifications, drug–host responses, and biomarker discovery, to name a few. While this long list of applications keeps growing every year, the isobaric tags methods are not immune from certain limitations. For instance, iTRAQ labeling may introduce an undesirable charge enhancement, which reduces the number of identifiable peptides. Reporter ion compression observed in complex biological samples can lead to less reliable quantification, especially for low abundance proteins. Their high production cost also prevents many researchers from adopting these powerful technologies. Although DiART reagents cannot solve all problems associated with isobaric labeling, at least they are a cost-effective alternative of expensive iTRAQ and TMT. Furthermore, DiART reagents are less susceptible to the level-off effect of reporter ions, thanks to their easy fragmentation. As a result, DiART would provide an additional way to alleviate the underestimation of protein quantification, complementing other efforts to minimizing iTRAQ ratio distortion.

### AUTHOR INFORMATION

**Corresponding Author**

*E-mail: sli@umd.edu (S. Li); sijiliu@genomics.org.cn (S. Liu).*

2914

dx.doi.org/10.1021/ac203467q1 Anal. Chem. 2012, 84, 2908−2915
Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Z.C. and Q.W. contributed equally to this work. This work was supported by the National Basic Research Program of China (973 Project).

■ REFERENCES


(6) Gan, C. S.; Chong, P. K.; Pham, T. K.; Wright, P. C. J. Proteome Res. 2007, 6, 821–827.


