

# Assessment of TMT Labeling Efficiency in Large-Scale Quantitative Proteomics: The Critical Effect of Sample pH

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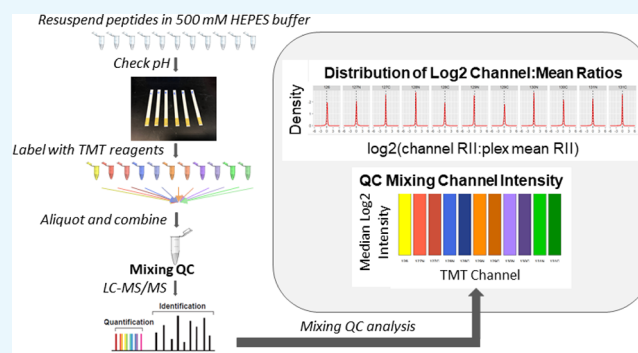
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**ABSTRACT:** Isobaric labeling via tandem mass tag (TMT) reagents enables sample multiplexing prior to LC–MS/MS, facilitating high-throughput large-scale quantitative proteomics. Consistent and efficient labeling reactions are essential to achieve robust quantification; therefore, embedded in our clinical proteomic protocol is a quality control (QC) sample that contains a small aliquot from each sample within a TMT set, referred to as “Mixing QC.” This Mixing QC enables the detection of TMT labeling issues by LC–MS/MS before combining the full samples to allow for salvaging of poor TMT labeling reactions. While TMT labeling is a valuable tool, factors leading to poor reactions are not fully studied. We observed that relabeling does not necessarily rescue TMT reactions and that peptide samples sometimes remained acidic after resuspending in 50 mM HEPES buffer (pH 8.5), which coincided with low labeling efficiency (LE) and relatively low median reporter ion intensities (MRIIs). To obtain a more resilient TMT labeling procedure, we investigated LE, reporter ion missingness, the ratio of mean TMT set MRIT to individual channel MRIT, and the distribution of log<sub>2</sub> reporter ion ratios of Mixing QC samples. We discovered that sample pH is a critical factor in LE, and increasing the buffer concentration in poorly labeled samples before relabeling resulted in the successful rescue of TMT labeling reactions. Moreover, resuspending peptides in 500 mM HEPES buffer for TMT labeling resulted in consistently higher LE and lower missing data. By better controlling the sample pH for labeling and implementing multiple methods for assessing labeling quality before combining samples, we demonstrate that robust TMT labeling for large-scale quantitative studies is achievable.



## INTRODUCTION

Proteomics has become a powerful tool in biological research; however, limitations remain regarding throughput, proteome coverage, and quality of quantitation.<sup>1</sup> In recent years, the development and refinement of isobaric labeling strategies—a method of chemically derivatizing proteins and peptides with isobaric chemical tags—have helped to address some of these limitations. A specific class of isobaric labels known as tandem mass tags (TMTs) are compounds composed of (1) a mass reporter region, (2) a cleavable linker region, (3) a mass normalization region, and (4) an amine-reactive group<sup>2</sup> (Figure 1A). In multiplex TMT experiments, peptides from individual samples are tagged with a TMT labeling reagent with a unique reporter ion. While the different TMT reagents are isobaric, they differ in how the heavy isotopes are distributed on the mass reporter and mass normalization regions. TMTs are designed so that the mass reporter region is cleaved at a linker region upon high-energy collision-induced dissociation (HCD).<sup>1,2</sup> From the combined samples, the intensity of distinct fragment reporter ions at different *m/z* values is detected in the tandem mass spectra and is used to

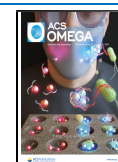
determine the contribution of peptides from each channel (sample), enabling a relative quantification of the peptides across samples in a single LC–MS run.<sup>2</sup> TMT labeling is invaluable to quantitative proteomics, and a major advantage is that this multiplexing strategy allows for higher throughput for quantitative analyses with deep proteome coverage. Once samples from a TMT set are labeled, they can be combined into a single sample for subsequent processing and analyses.<sup>3,4</sup>

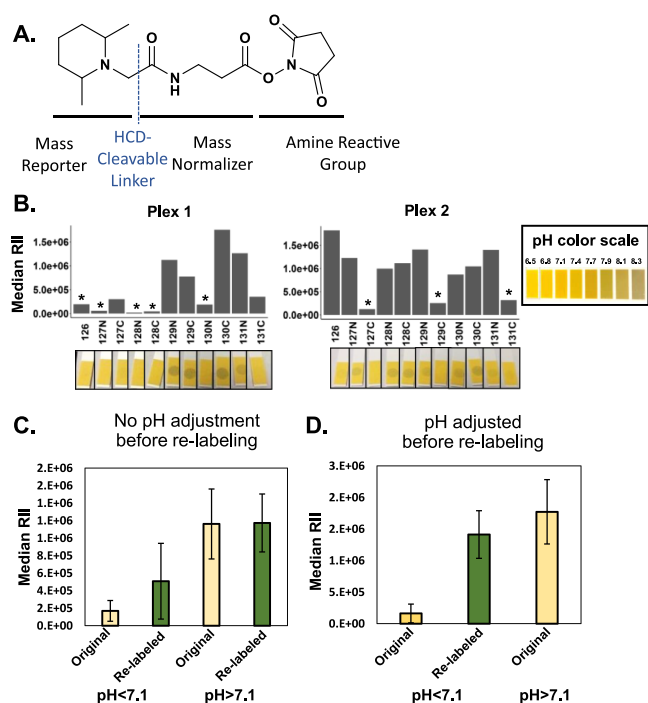
Recent advances have validated the use of additional TMT reagents, which currently allows for up to 16 samples per TMT set.<sup>1</sup> While the cost of TMT reagents has been viewed as a drawback, recent protocol adaptations now allow labeling experiments to use as little as one-eighth of the recommended reagents without sacrificing labeling efficiency (LE) or

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**Figure 1.** Sample pH is correlated with MRIIs of TMT-labeled samples. (A) Diagram of the general structure and relevant regions of a TMT reagent. (B) MRII values generated from Mixing QC samples and the corresponding pH results from two sets of peptides labeled with TMT-11 reagents. Channels marked with an asterisk indicate an MRII ratio  $>3$ . Images of pH strips were taken after blotting  $1\ \mu\text{L}$  of sample onto ColorpHast Strips, pH 6.5–10.0 (Sigma). (C,D) MRII values of TMT-11-labeled peptide samples with pH values  $<7.1$  or  $\geq 7.1$  before and after relabeling. Samples were either relabeled without adjusting pH (C) or were adjusted to ensure all samples had pH  $> 7.1$  prior to relabeling (D).

identifications.<sup>5</sup> On the analysis side, if several biological samples can run simultaneously in an LC–MS run, the number of data sets, run-to-run variation, and missing values of low abundant peptides are reduced.<sup>6,7</sup> Furthermore, isobaric labeling enables experiments where sample amounts might be limiting, as the total peptide signal from all channels triggers MS/MS fragmentation for peptide identification. Using this feature, a strategy known as “BASIL” (boosting to amplify the signal in isobaric labeling) can be applied, in which biologically similar material is added to a “boost” channel in each multiplex and thereby amplifies the signal for deeper coverage.<sup>8–10</sup>

While there are numerous benefits of TMT labeling, technical aspects of the associated protocols can present challenges. One major issue that can arise is the robustness of the labeling step because of the potential poor reaction efficiency between TMT labels and peptides. Incomplete reactions in specific channels can jeopardize downstream quantitative analysis, as unlabeled peptides in individual samples will not yield TMT reporter ion intensities for those samples. To address this concern, researchers often check for poor LE in a Mixing quality control (QC) test of samples before combining and further processing of samples, but bad labeling reactions still result in significant time and reagent loss as samples need to be relabeled and then analyzed again via LC–MS/MS.<sup>11</sup> Furthermore, when relabeling is ineffective, irreplaceable samples can be lost. A recent protocol developed through the Clinical Proteomics Tumor Analysis Consortium

seeks to provide a standardized TMT-11 workflow for use across proteomics laboratories.<sup>11</sup> While this processing pipeline in conjunction with the reduced TMT reagent protocol<sup>5</sup> is relatively robust, we have encountered situations where samples from different experiments had poor labeling efficiencies. In this work, we report the sample pH as a primary cause of poor LE and the way to prevent such failed TMT labeling, as well as a set of metrics for assessing LE.

## RESULTS AND DISCUSSION

While it is possible to determine the quality of TMT labeling for individual channels by taking a small aliquot of each labeled sample from a multiplex TMT set and analyzing it by LC–MS/MS, this would require significant instrument analysis time. Thus, in this work, we opted to utilize the concept of a single Mixing QC sample established in a well-validated protocol,<sup>11</sup> and we further demonstrate here that this sample can provide the necessary information to accurately determine channel-level LE when analyzed as described.

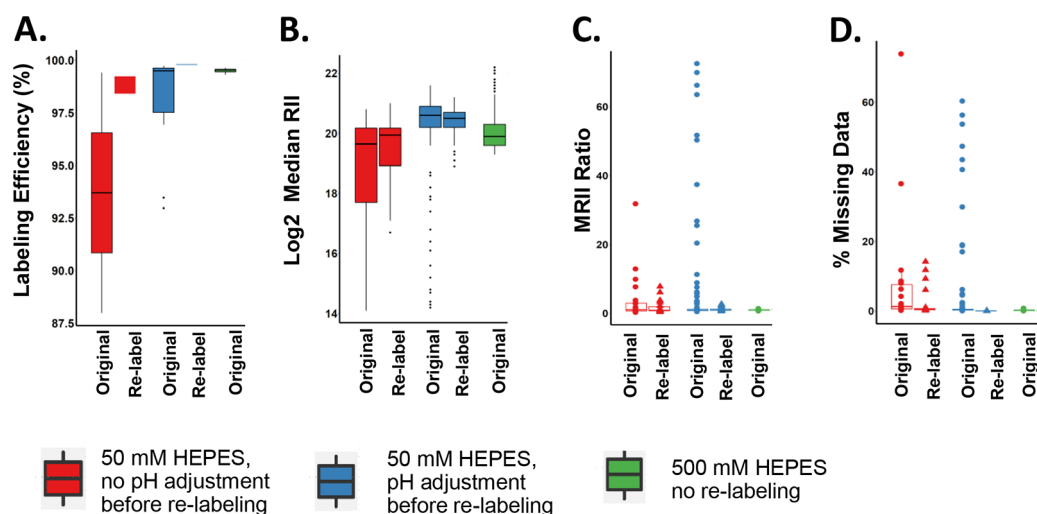
Our experimental results are derived from multiple projects and include data from different sample types, including MCF10A cell pellets, human-derived cell pellets from bone marrow aspirate, peripheral blood, and leukapheresis, and cryopulverized rat kidney and lung tissue. In all cases, a standard protocol was used to extract and digest proteins and label peptides with TMT reagents; therefore, the sample source is not critical to the interpretation of TMT labeling results.

### Sample pH Is a Critical Factor of Labeling Efficiency.

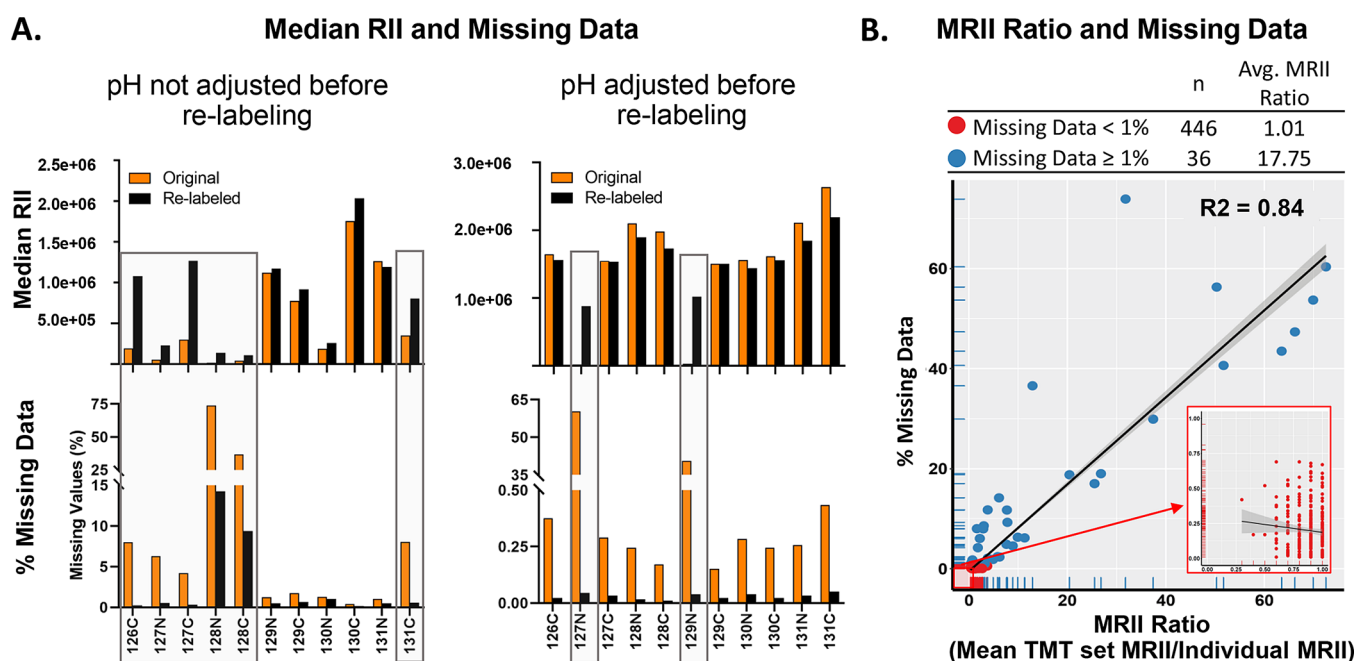
During the implementation of the standard protocol paired with reduced TMT reagent<sup>5,11</sup> where dried peptides were redissolved in 50 mM HEPES (pH 8.5), we sometimes observed failed or poor TMT labeling (Figure 1B). The data here were from two sets of MCF10A cell pellets labeled with TMT-11 reagents, as described in the original labeling method. The LE was 87.99% for the first TMT-11 set and 99.41% for the second TMT set. Based on the median reporter ion intensity (MRII), it was clear that many channels were labeled poorly compared to those with high MRII.

During the troubleshooting process, we hypothesized that the sample pH was altered because before TMT labeling, the samples had been dried down from an acidic SPE elution solution (50% ACN; 0.1% FA). Any residual acid from incomplete drying could alter the sample pH. After testing the pH of the samples resuspended in 50 mM HEPES (pH 8.5), all samples that had failed TMT labeling reactions had pH values less than 7, and five of the eight channels from the two TMT sets remained poorly labeled even after attempting to relabel without adjusting the pH, as indicated by comparatively low MRII values (Figure 1B,C).

In another independent study, 11 sets of samples of the digested cell pellets derived from human bone marrow aspirate, peripheral blood, or leukapheresis were labeled with TMT-11 reagents using the original method described. We again observed some TMT sets with lower than expected labeling efficiencies, ranging from 92.97% to 99.74%, and 20 of 121 samples (or TMT channels) had failed labeling reactions. All samples with poor reactions (as indicated by comparatively low MRII values) had pH values below 7.1, while 20 randomly chosen samples with apparently successful labeling reactions all had a pH greater than or equal to 7.1. The 20 samples with poor labeling were pH adjusted by adding the additional HEPES buffer of a higher concentration, with final



**Figure 2.** Measurements of TMT labeling quality in experiments with different buffer conditions. Data from Mixing QC samples derived from three representative experiments where TMT labeling was performed with different buffer conditions: peptides resuspended in 50 mM HEPES for labeling and no pH adjustment prior to relabeling (red boxes and markers); peptides resuspended in 50 mM HEPES for labeling and samples were adjusted to pH > 7.1 prior to relabeling (blue boxes and markers); peptides resuspended in 500 mM HEPES for labeling, and no relabeling was necessary for any samples (green boxes and markers). From Mixing QC samples generated in each set of experiments, the following metrics were calculated to measure the quality of TMT labeling: (A) LE, (B) log<sub>2</sub> MRR values, (C) MRR ratio, and (D) percent missing data.



**Figure 3.** TMT reporter ion intensities and missing data are impacted by sample pH during labeling. (A) MRRIs and the corresponding missing data from two TMT-11 multiplexes containing channels with poor labeling (these samples are outlined and indicated with gray shading). Peptide samples were relabeled without pH adjustment (left plots) or were pH adjusted before relabeling (right plots). (B) Correlation between missing data and MRR ratio for samples across multiple experiments; “n” represents the number of channels used to make the missing data and MRR ratio calculations.

concentrations of HEPES ranging from 45 to 310 mM in samples before relabeling. Before relabeling, samples with a pH below 7.1 had an average MRR of  $2 \times 10^5$ , but after pH adjustment and relabeling of 20/121 samples, the average MRR for all samples was  $1 \times 10^6$ . In particular, for the 20 samples that were pH adjusted and relabeled, MRR increased over 5-fold (*t*-test,  $p < 0.001$ ). Furthermore, the samples that originally had pH values above 7.1 had an average MRR that was 11× higher than the samples with pH values below 7.1 (Figure 1D, *t*-test,  $p < 0.001$ ). Adjusting the pH before

relabeling resulted in an LE >99% for all TMT sets and successfully rescued labeling reactions (Figures 1D and 2A–D).

**Improved Protocol with Higher Buffer Capacity.** We hypothesized that the significant variability in sample pH is due to trace amounts of acid remaining after vacuum centrifugation of peptide samples prior to resuspension for labeling and the limited buffer capacity of 50 mM HEPES (pH 8.5) for peptide solubilization. Additionally, the reduced amount of TMT label<sup>5</sup> could make the reactions more susceptible to failure.



Moreover, the protocol used by Li et al.<sup>1</sup> to validate the TMT-pro reagents used 200 mM HEPES (pH 8.5) to resuspend samples, supporting the use of a higher concentration of HEPES (pH 8.5) buffer in TMT labeling reactions. Using a higher concentration of HEPES buffer is a simple method to increase buffer capacity and to prevent poor labeling caused by variability in sample pH, which would prevent the loss of time, resources, and precious samples from relabeling or repeating whole experiments.

Subsequently, we implemented a revised protocol with 500 mM HEPES (pH 8.5) in place of 50 mM HEPES (pH 8.5) to resuspend peptide samples derived from cryopulverized rat kidney and lung tissue before relabeling. We used this revised protocol to label 209 samples (19 TMT-11 sets). The LE of each set was above 99.3%, indicating that the new protocol successfully safeguards the samples against poor labeling reactions (Figure 2A–D). The complete, revised protocol is provided in the Supplemental Methods section of the Supporting Information.

**Poor Labeling Results in Higher MR II Ratios, Increased Data Missingness, and Broadened Ratio Distributions.** Because calculating the LE for the entire TMT experimental set does not effectively identify individual channels that might be poorly labeled, we explored several methods to assess LE at the individual channel level for each TMT set.

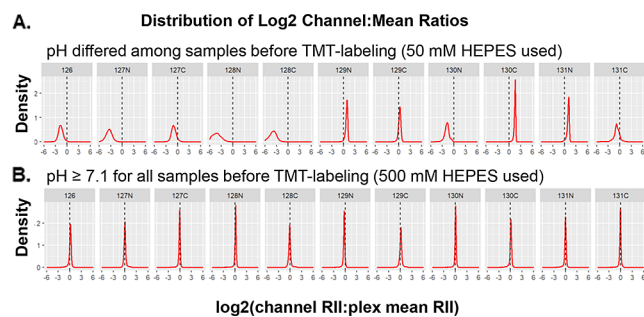
Intuitively, comparatively low MR II values for an individual channel in a TMT set is a red flag that there could potentially be a labeling issue. To quantify this, we calculated an MR II ratio by dividing the mean MR II of the TMT set by the MR II from an individual sample (calculations described in more detail in the Methods section), where a value greater than 1 would indicate that the individual channel has a lower MR II as compared to the TMT set as a whole.

While calculating MR II ratios is a quick way to determine the labeling quality, we implemented a complementary approach to assess the downstream impact of poor labeling by calculating the percentage of missing data (calculations described in more detail in the Methods section). Overall, the percentage of missing data was notably high for samples with comparatively low MR II values and therefore higher MR II ratios ( $R^2 = 0.84$ ; Figures 2C,D and 3A,B). For channels with MR II ratios above 3, missing data averaged  $19.6\% \pm 21.2\%$ , while channels with MR II ratios below 3 averaged  $0.27\% \pm 0.65\%$  ( $t$ -test,  $p < 0.001$ ). The high correlation of the MR II ratio to missing data percentage illustrates the utility of the MR II ratio for determining if an individual channel should be relabeled to prevent missing data and issues with later analyses.

In particular, the samples from the first two TMT-11 sets in which we used 50 mM HEPES before TMT labeling with MR II ratios above 3 had missing data averaging  $7.69\% \pm 16.39\%$  (Figure 2C,D), and individual channels with relatively low MR II values had notably high missing data percentages (Figure 3A). While relabeling of these TMT-11 sets reduced missing data to  $2.24\% \pm 4.02\%$ , five of the eight channels from the two sets remained poorly labeled. After pH adjustment and relabeling, the 20 poorly labeled samples from the 11 TMT-11 sets had an average missing data percentage that improved from  $3.66\% \pm 11.45\%$  to  $0.04\% \pm 0.02\%$  (Figure 2D), and individual channels that were pH adjusted before relabeling showed both relatively higher MR II values and lower missing data after relabeling (Figure 3A). For the 19 sets of TMT-11 labeled samples that were resuspended in 500 mM HEPES

(pH 8.5) before labeling, MR II ratios averaged 1 and missing data averaged less than 0.25%, further validating that the higher concentration of HEPES before TMT labeling is an effective tool for preventing variability in labeling quality (Figure 2C,D). It has previously been described that TMT tags can label additional amino acid side chains (besides *N*-terminal residues and internal K residues, which are typically viewed as TMT targets and included in our MS-GF+ searches of dynamic TMT modification).<sup>5</sup> Thus, we set up additional MS-GF+ searches on selected Mixing QC data sets to evaluate the presence of TMT modifications to S, T, Y, and H residues and assess the impact of sample pH on the labeling of these amino acids. While TMT modification of internal S, T, Y, or H residues was detected on identified peptides as frequencies ranging from 3% to 10%, the levels of missing RII data for these peptides were consistent with the levels of missing data calculated from our standard MS-GF+ searches, and samples with low pH prior to labeling showed high levels of missing data (Figure S1). Importantly, when the sample pH was adjusted prior to relabeling, the levels of missing data decreased. Thus, these data indicate that the sample pH influences all TMT labeling reactions equivalently and does not result in altered amino acid selectivity.

Finally, we utilized the ratio distribution plots for each TMT experimental set to assess the robustness of quantification. These plots are generated from the Mixing QC test samples (Figures 4 and Figure S2). In large-scale studies that compare



**Figure 4.** Additional measures of TMT labeling quality from Mixing QC samples. Distributions of ratios calculated by log<sub>2</sub> transforming the values of (channel RII/plex mean RII) for each peptide identified from Mixing QC samples. (A) Peptide aliquots resuspended in 50 mM HEPES prior to labeling, with numerous channels later revealed to be pH < 7.1. (B) Peptide aliquots resuspended in 500 mM HEPES (pH 8.5) prior to labeling.

samples across multiple TMT sets and use a common universal reference in one channel of each set, distribution plots can be generated by calculating the peptide intensity ratios of each channel to the reference channel of that set. For these plots, however, we opted to use the mean RII of each peptide identified in a TMT set as the common denominator by which we divided each sample. This ratio is then log<sub>2</sub>-transformed, and the distribution of the log<sub>2</sub>-transformed ratios for each channel within a set is plotted. In general, when channels are loaded with an equivalent amount of peptide and labeling occurs equally efficiently, we would expect most peptides to have relatively comparable RII values in each channel. As such, the expected distribution plot for each channel should have a narrow peak centered around zero (a reporter ion intensity ratio of 1 will yield a value of zero when log<sub>2</sub> transformed). When individual channels in a TMT set yield peaks that are

shifted to the left or right, this indicates that the signals detected in this channel are lower or higher than the TMT set means. If labeling is still efficient in these differentially loaded samples, distribution plot peaks remain sharp despite not being centered around zero. In these experiments, however, poorly labeled samples yielded peaks that are both left-shifted (i.e., ratios of less than 1) and were significantly broader in their distribution profiles (Figure 4). This widening of the peak is due to the differential LE of peptide species, which results from suboptimal labeling conditions—rather than a uniformly lower signal that would be expected from lower peptide loadings. Thus, poor labeling can be identified by the resulting wide range of TMT RII. This has a compounding effect when considered alongside the issue of missing data because not only are a large proportion of peptides not identified in certain channels, but the peptides that are identified have reporter ion intensities that are much more variable and quantitatively unreliable. While the poor quantification brought on by inefficient labeling can be partially accounted for using standard proteomics data processing steps such as median centering, the reduced measurement precision leads to reduced statistical power in downstream analysis.

## CONCLUSIONS

Efficient, robust, and consistent TMT labeling reactions are critical to obtaining accurate quantitative proteomic measurements. TMT LE is highly dependent on pH; therefore, using our protocol with a higher concentration of HEPES (pH 8.5) buffer (e.g., 200–500 mM) is an easy and cost-effective way to safeguard against poor labeling reactions. Although not tested here, we expect that these findings related to the effect of buffer pH on labeling quality are relevant to other NHS-based labeling reagents (such as iTRAQ). Furthermore, we have shown that MRRI ratios and missing data calculated from Mixing QC samples are tightly correlated and useful indicators of TMT labeling quality. In particular, plotting the distribution of log<sub>2</sub> reporter ion ratios from Mixing QC samples can help differentiate between unequal peptide loading (sharp peaks shifted to the left or right) and TMT LE (left-shifted peaks that are significantly broader), making it a particularly robust approach for determining the proper steps to take to remedy individual samples before moving forward and generating data that are irreversibly flawed.

## METHODS

**Protein Digestion and Peptide Desalting.** The data presented in this work come from multiple experiments in our laboratory, which utilize proteins from different biological sources, including MCF10A cell line pellets, human-derived cell pellets from bone marrow aspirate, peripheral blood, and leukapheresis, and cryopulverized rat kidney and lung tissue. Since the LE assessments are independent of specific sample types, specific sample details are not provided here. All samples were extracted and digested as detailed in the Mertins et al. 2018 workflow.<sup>11</sup> In summary, the samples were lysed and denatured in 8 M urea solution with protease and phosphatase inhibitors. To reduce denatured proteins, the samples were treated with dithiothreitol (5 mM, 1 h in a thermomixer set to 37 °C with shaking at 1000 rpm) and then alkylated with iodoacetamide (10 mM, 45 min in a thermomixer set to 25 °C with shaking at 1000 rpm in the dark). Proteins were then digested with Lys-C (Wako, 1 mAU per 50 μg total protein)

for 2 h at 25 °C in a thermomixer with shaking at 850 rpm, followed by trypsin (1 μg per 50 μg total protein; Promega) for 14 h at 25 °C in a thermomixer with shaking at 850 rpm. Desalting of the peptide samples was performed using tC18 SepPak, 100 mg SPE cartridge (Waters WAT036820). Peptide concentrations were determined using bicinchoninic acid (BCA) assay (Pierce), and the samples were aliquoted for the desired peptide mass and dried via vacuum centrifugation in preparation for TMT labeling.

### TMT Labeling and Mixing QC Sample Preparation.

Peptide samples ranging from 120 to 400 μg were reconstituted in 50 mM HEPES (pH 8.5) to a concentration of 5 μg/μL. TMT-10 reagent (ThermoFisher Scientific cat# 90111) and TMT-11 reagent (ThermoFisher Scientific Cat# A37724) were resuspended in anhydrous ACN to a concentration of 20 μg/μL. The appropriate TMT reagent was added to each sample at 1:1 reagent/peptide (wt/wt), mixed, and incubated for 1 h at 25 °C. The labeled samples were diluted to 2.5 mg/mL with 50 mM HEPES (pH 8.5), 20% ACN. For the Mixing QC sample, 2 μg aliquots of each labeled sample were combined for each plex, dried via vacuum centrifugation, and desalted using a C18 Stage-Tip method (Supplemental Methods in Supporting Information). The samples were resuspended in 40 μL of 3% ACN; 0.1% FA was then diluted to 0.1 μg/μL with water after BCA assay for LC–MS/MS analysis.

**LC–MS/MS Analysis.** Mixing QC samples were separated using a Waters nano-Aquity UPLC system (Waters) with an in-house 75 μm i.d. × 70 cm length C18 column packed with 3 μm Jupiter particles (Phenomenex). A 100 min gradient of 100% mobile phase A (0.1% (v/v) FA in water) to 60% (v/v) mobile phase B (0.1% (v/v) FA in ACN) was applied. This system was paired with a Thermo Q-Exactive Plus or equivalent mass spectrometer for MS/MS analysis. MS spectra were collected from 300 to 2000 *m/z* at a mass resolution of 50 000 or greater. MS<sub>2</sub> spectra were collected in a data-dependent fashion and fragmented with HCD; +1 charged species were excluded, and the dynamic exclusion window was 30 s. All MS<sub>2</sub> spectra were collected at a mass resolution of 35 000 or greater. Complete detailed information about mass spectrometry settings is provided in the Supporting Information.

**Data Processing and Peptide Identification.** Quantitative TMT LC–MS/MS data were extracted using a previously described method.<sup>12</sup> Data were preprocessed with DeconMSn<sup>13</sup> and DtaRefinery<sup>14</sup> for recalibration of parent ion *m/z*. Calibrated spectra were searched against the appropriate protein sequence databases (Human RefSeq release version 37 or Rat RefSeq database downloaded in April 2018, each supplemented with common contaminants including trypsin and keratin sequences) using the MS-GF+ tool.<sup>15</sup> For the determination of peptide labeling efficiencies, MS-GF+ parameters were set to include TMT as a variable modification on lysine residues and *N*-terminal amines. Carbamidomethylated cysteine was set as a fixed modification, and oxidation of methionine was set as a dynamic modification. The intensities of all TMT reporter ions from peptide spectrum matches were extracted using MASIC software.<sup>16</sup> The results were filtered to a 1% false discovery rate at the peptide level by adjusting MS-GF+ peptide *Q*-value cutoffs and employing a target-decoy approach using reversed protein sequences.

**Calculating Labeling Efficiency, MRRI Ratios, and Missing Data.** Using the data sets generated from Mixing

QC samples, the overall LE of each TMT set was calculated according to the following formula:

$$\frac{(((\text{Total unique peptide IDs}) - (\text{Unique peptide IDs without TMT label})) / (\text{Total unique peptide IDs})) \times 100}{\text{MREI ratios were calculated as follows:}}$$

MREI ratios were calculated as follows:

$$\text{Mean TMT set MREI/individual channel MREI}$$

Missing data for individual channels were calculated according to the following formula:

$$\frac{((\text{Unique peptide IDs missing MREI values in channel}) / (\text{Total unique peptide IDs in plex})) \times 100}{\text{Generating Ratio Distribution Plots.}}$$

Using the data sets generated from Mixing QC samples for each TMT set, reporter ion intensities in each TMT channel were aggregated to the peptide level. Mean values were then calculated for each identified peptide across all TMT channels, and the MREI values for each channel were then divided by this mean. The resulting ratios were log<sub>2</sub>-transformed and used to make distribution plots for each TMT channel within a plex.

**Assessment of Mixing QC Labeling.** We considered TMT labeling to be successful if (1) LE is greater than 99% and (2) there is less than a 3-fold difference in an individual channel MREI to the average plex MREI, referred to as the “MREI ratio.” While the MREI ratio would shrink in TMT experimental sets with multiple failed channels because the average plex MREI would also decrease, overall this metric correlates well with other assessments of poor labeling (discussed further in the Results and Discussion section) and is a method to compare labeling quality across TMT experiments where MREI values can vary widely. Relabeling is routinely performed if the labeling reaction appears to be unsuccessful based on these metrics.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c00776>.

Sample pH influences the labeling of numerous amino acid side chains with TMT reagents (Figure S1); robustness of improved TMT labeling protocol as measured by mixing QC sample ratio distribution plots (Figure S2); and improved TMT labeling and mixing QC protocol and LC-MS/MS analysis (Supplemental Methods) (PDF)

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

BCA	bicinchoninic acid
BSA	bovine serum albumin
DTT	dithiothreitol
FDR	false discovery rate
HPLC	high-performance liquid chromatography
IAA	iodoacetamide
LE	labeling efficiency
MREI	median reporter ion intensity
<i>m/z</i>	mass-to-charge ratio
MS	mass spectrometry
PSMs	peptide-to-spectrum matches
SPE	solid-phase extraction
TMT	tandem mass tag

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