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TITLE:

High-throughput and Deep-proteome Profiling by 16-plex Tandem Mass Tag Labeling Coupled with Two-dimensional Chromatography and Mass Spectrometry

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SUMMARY:

Presented here is an optimized high-throughput protocol developed with 16-plex tandem mass tag reagents, enabling quantitative proteome profiling of biological samples. Extensive basic pH fractionation and high-resolution LC-MS/MS mitigate ratio compression and provide deep proteome coverage.

ABSTRACT:

Isobaric tandem mass tag (TMT) labeling is widely used in proteomics because of its high multiplexing capacity and deep proteome coverage. Recently, an expanded 16-plex TMT method has been introduced, which further increases the throughput of proteomic studies. In this manuscript, we present an optimized protocol for 16-plex TMT-based deep-proteome profiling, including protein sample preparation, enzymatic digestion, TMT labeling reaction, two-dimensional reverse-phase liquid chromatography (LC/LC) fractionation, tandem mass spectrometry (MS/MS), and computational data processing. The crucial quality control steps and improvements in the process specific for the 16-plex TMT analysis are highlighted. This multiplexed process offers a powerful tool for profiling a variety of complex samples such as cells, tissues, and clinical specimens. More than 10,000 proteins and posttranslational modifications such as phosphorylation, methylation, acetylation, and ubiquitination in highly complex biological samples from up to 16 different samples can be quantified in a single experiment, providing a potent tool for basic and clinical research.

INTRODUCTION:

Rapid developments in mass spectrometry technology have enabled to achieve high sensitivity and deep proteome coverage in proteomics applications[1,2]. Despite these developments, sample multiplexing remains the bottleneck for researchers handling the analysis of a large sample cohort.

Multiplexed isobaric labeling techniques are extensively used for proteome-wide relative quantitation of large batches of samples[3-6]. Tandem mass tags (TMT)-based quantitation is a popular choice for its high multiplexing capability[7,8]. TMT reagents were initially launched as a 6-plex kit capable of quantifying up to 6 samples simultaneously[9]. This technology was further expanded to quantify 10-11 samples[10,11]. Recently developed 16-plex TMTpro (termed TMT16 hereafter) reagents have further increased the multiplexing capacity to 16 samples in a single experiment[12,13]. The TMT16 reagents use a proline-based reporter group, whereas 11-plex TMT applies a dimethylpiperidine-derived reporter group. Both TMT11 and TMT16 use the same amine reactive group, but the mass balance group of TMT16 is larger than that of TMT11, enabling the combination of 8 stable C13 and N15 isotopes in the reporter ions to achieve 16 reporters (**Figure 1**).

The increase in multiplexing capability provides a platform for designing experiments with sufficient replicates to overcome statistical challenges[14]. Furthermore, the additional channels in the 16-plex TMT help reduce the total amount of starting material per channel, which may aid in the development of emerging single-cell proteomics[15]. The high multiplexing capacity will also be valuable in quantitation of post-translational modifications, which typically requires high amounts of starting material[16,17].

Proteomic workflows employing TMT technology have been streamlined[18-20], and they have evolved significantly over the past decade in terms of sample preparation, liquid chromatography separation, mass spectrometric data acquisition, and computational analysis[21-26]. Our previous article provides an in-depth overview of the 10-plex TMT platform[27]. The protocol described here introduces a detailed, optimized method for TMT16, including protein

extraction and digestion, TMT16 labeling, sample pooling and desalting, basic pH, and acidic pH reverse phase (RP) LC, high-resolution MS, and data processing (**Figure 2**). The protocol also highlights the key quality control steps that have been incorporated for successfully completing a quantitative proteomics experiment. This protocol can be routinely used to identify and quantify greater than 10,000 proteins with high reproducibility, to study biological pathways, cellular processes, and disease progression[20,28-30].

PROTOCOL:

Human tissues for the study were obtained with approvals from the Brain and Body Donation Program at Banner Sun Health Research Institute.

1. Protein extraction from tissue and quality control

NOTE: To reduce the impact of sample harvesting on the proteome, it is crucial to collect samples in minimal time at low temperature if possible[31]. This is especially important when analyzing posttranslational modifications as they typically are labile, for example, some phosphorylation events only have few seconds of half-life[32,33].

1.1. Excise and weigh tissue samples

1.1.1. Tare a 1.5 mL microcentrifuge tube using an analytical balance and pre-cool the tube over dry ice.

1.1.2. Cut a frozen tissue (e.g., human brain tissue, ~10 mg) from a defined region into small pieces and transfer the tissue pieces into the pre-cooled tube immediately.

NOTE: To reduce sample heterogeneity, it is important to use homogenous sizes and anatomic regions for all 16 samples. The amount of protein obtained from the tissue is typically 5-10% of tissue weight.

1.1.3. Weigh the tube along with the tissue and put the tube immediately on dry ice. Process the remaining 15 samples using the same procedure. Keep the samples in liquid nitrogen immediately after dissection, and store at -80 °C.

1.2. Lyse tissue samples

1.2.1. Prepare fresh lysis buffer (50 mM HEPES pH 8.5, 8 M urea, and 0.5% sodium deoxycholate) on the day of the experiment. Phosphatase inhibitors should be added into the lysis buffer to preserve the phosphorylation state of proteins.

NOTE: Put the lysis buffer at room temperature before using it, as 8 M urea will precipitate on ice, which may result in incomplete protein denaturation during sample lysis and reduce protein digestion efficiency.

1.2.2. Add the lysis buffer (add 100 μL lysis buffer per 10 mg tissue to achieve a final protein concentration of 5 to 10 μg/μL) and glass beads (~20% of the lysate volume, 0.5 mm diameter) to each sample.

1.2.3. Lyse the tissue in a blender at 4 °C with a speed setting 8 for 30 s, rest for 5 s, repeat until the samples are homogenized (~ 5 cycles).

1.3. Prepare aliquots of the lysates.

1.3.1. Prepare at least two aliquots for each sample. A small aliquot (~10 μL) is used for the analysis of protein concentration and the evaluation of protein quality (e.g., western blotting validation of positive-control proteins). A larger aliquot (~50 μL) is used for proteome analysis.

1.3.2. Freeze the aliquots immediately on dry ice, and store at -80 °C until further use.

1.4. Measure protein concentration

NOTE: Protein concentration can be measured by the BCA assay or short SDS gel staining method[34](**Figure 3A**). Because the non-protein reducing components in the tissue lysate may affect the measurement in the BCA assay, users may validate the protein concentration by the short SDS gel staining method. The short SDS gel staining method is presented here.

1.4.1. Dilute 16 aliquoted samples by 10-fold and prepare the BSA standard (e.g., BSA titrations of 0.15, 0.5, 1.5 and 3 μg).

1.4.2. Run the samples and the BSA standard on a 10% SDS-PAGE gel (24 well) with a stacking gel until all proteins migrate approximately 3 mm into the gel.

1.4.3. Stain the gel with Coomassie blue for 1 h, and de-stain the gel until the background in the blank region is clear.

1.4.4. Scan the gel to measure the intensities of Coomassie-stained protein bands by ImageJ and create a BSA standard curve according to the measurements.

1.4.5. Calculate the absolute protein concentration by the standard curve.

NOTE: The final protein concentration for each sample in this experiment was ~5-10 μg/μL. For TMT16-based proteomics analysis, 50 μg protein per sample (total 0.8 mg protein for whole-proteome analysis) is sufficient.

1.5. Sample quality control

NOTE: This quality control step is critical to identify low-quality samples before performing the TMT analysis. For samples with known protein change, it is suggested to validate the change by

western blotting. Standard SDS-PAGE analysis is also recommended to examine protein patterns and exclude any samples with high degrees of degradation (**Figure 3B**).

1.5.1. Take ~10 μg of each sample from the small aliquot and run samples on a gradient SDS-PAGE gel until the bromophenol blue dye reaches the bottom of the gel.

1.5.2. Stain the gel with Coomassie blue, and de-stain the gel. Inspect the protein quality to remove highly degraded protein samples.

NOTE: Degraded samples can be identified as one that have very few protein bands in the high molecular weight region and intensified bands in the low molecular weight region (**Figure 3B**).

2. In-solution protein digestion, peptide reduction and alkylation, digestion efficiency test, and peptide desalting

2.1. Protein digestion by Lys-C and trypsin

2.1.1. Take ~50 μg protein from the large aliquot of each sample and add lysis buffer to 50 μL.

2.1.2. Add 100% acetonitrile (ACN) to reach to a final concentration of 10%.

2.1.3. Perform the Lys-C digestion by adding Lys-C at a protein:Lys-C ratio of 100:1 (w/w) and incubating at room temperature for 3 h.

2.1.4. Samples are diluted to contain a final concentration of 2 M urea by 50 mM HEPES (pH 8.5).

2.1.5. Add trypsin into each sample at a protein:trypsin ratio of 50:1 (w/w) and perform the digestion at room temperature for 3 h or overnight.

2.2. Peptide reduction and alkylation

2.2.1. Add freshly prepared dithiothreitol (1 M DTT) solution to a final 1 mM concentration and incubate for 1 h at room temperature to reduce the disulfide bonds.

2.2.2. Add freshly prepared iodoacetamide (1 M IAA) solution to a final 10 mM concentration for 30 min in the dark to alkylate cysteine residues.

2.2.3. Quench the unreacted IAA by adding 1M DTT to a final 30 mM concentration and incubate at room temperature for another 30 min.

2.3. Examine the digestion efficiency

2.3.1. Take ~1 μg of each sample and desalt using C18 resin-coated pipette tips according to the

manufacturer's protocol.

2.3.2. Analyze each sample by a short gradient LC-MS/MS run (see more details in step 5).

2.3.3. Perform a database search for MS raw data (see more details in step 6). Calculate the percentage of identified peptides with at least one trypsin miscleavage site. The percentage is usually below 15%.

2.3.4. If the percentage is larger than 15%, add additional trypsin to the samples to repeat the digestion.

2.3.5. After digestion, acidify the samples by adding TFA to 0.5% (v/v). Check the pH by a pH strip to ensure the pH is less than 3.

2.4. Peptide desalting

2.4.1. Centrifuge the acidified peptides at 21,000 *x g* for 10 min. Transfer the supernatants to a new tube.

2.4.2. Wash C18 desalting columns (~25 µL resin) twice with 250 µL of 100% methanol by centrifuging at 500 *x g* for 30 s.

NOTE: To reduce the loss of peptides during the desalting process, choose desalting columns with binding capacity matching the amount of input.

2.4.3. Wash the columns twice using 250 µL of elution buffer (60% ACN, 0.1% TFA) by centrifuging at 500 *x g* for 30 s.

2.4.4. Equilibrate the columns with 250 µL of equilibration and wash buffer (0.1% TFA) by centrifuging at 500 *x g* for 30 s.

2.4.5. Load samples on the pre-equilibrated columns. Let the samples bind to columns by spinning at 100 *x g* for 6 min. Ensure that all the solution has passed through the column.

2.4.6. Wash the columns three times with 250 µL of equilibration and wash buffer by centrifuging at 500 *x g* for 30 s.

2.4.7. Elute the peptides by adding 125 µL of elution buffer to each column and spinning at 100 *x g* for 3 min. Check that the column does not contain any leftover solution.

2.4.8. Dry the eluted peptides in a vacuum concentrator and store the peptides at -80 ˚C for future TMT labeling.

3. TMT16 labeling of peptides, labeling efficiency test, sample pooling, and labeled peptide

desalting

3.1. TMT16 labeling of peptides

3.1.1. Resuspend each desalted peptide sample in 50 µL of 50 mM HEPES (pH 8.5) by vortexing several times or ultrasonic dissolving followed by using a pH strip to verify the pH.

NOTE: The sample may be acidic if not dried completely before labeling, which negatively influence the labeling efficiency. Ensure that pH is between 7 and 8.

3.1.2. Take ~1 µg of unlabeled peptides from each sample as negative controls for the TMT labeling efficiency test.

3.1.3. Dissolve TMT16 reagents in anhydrous ACN. Perform the labeling reaction by adding the reagents at a TMT:protein ratio of 1.5:1 (w/w) and incubating at room temperature for 30 min.

NOTE: The TMT:protein ratio used for TMT16 is 50% higher than the ratio used for TMT11. This small discrepancy may be due to the molecular mass of TMT16 being bigger (1.2-fold) than that of TMT11 reagents. The protein amount is estimated from the samples without considering the loss during desalting.

3.2. Labeling efficiency test

3.2.1. Take ~1 µg of labeled peptides from each sample for the labeling efficiency test. Put the remaining samples at -80 ˚C without quenching the reaction.

3.2.2. Desalt ~1 µg each of TMT16-labeled and unlabeled samples by C18 resin-coated pipette tips according to the manufacturer's protocol.

3.2.3. Analyze the samples by LC-MS/MS (see section 5, with the exception that the gradient is 10 min).

3.2.4. Estimate the labeling efficiency by analyzing the MS1 intensity reduction of unlabeled peptides between unlabeled and labeled samples. For complete labeling, select 6 to 10 different peptides to verify the labeling efficiency. Ensure that all peptides are labeled and unlabeled peptides are not observed.

NOTE: It is important to ensure the complete labeling of all samples for downstream accurate protein identification and quantification.

3.2.5. If the labeling is not complete, add additional TMT reagents to label the remaining peptides and check the labeling efficiency again before quenching. After the sample is completely labeled, quench the reaction at room temperature by adding hydroxylamine to a final 0.5% concentration and incubate at room temperature for 15 min.

3.3. Sample pooling and desalting

3.3.1. Pool half of each TMT-labeled sample to make a mixture.

3.3.2. Take 1 µg from the mixture and desalt by C18 resin-coated pipette tips, then analyze by LC-MS/MS using a short gradient (~30 min).

3.3.3. Calculate the relative concentration using the average intensity of each TMT16 reporter ion and comparing the discrepancies between the 16 channels. To achieve equal mixing of each channel, add the remainder of the TMT-labeled samples into the mixture according to the calculated average intensity. Repeat the adjustment until all samples are equally mixed. Representative data showing the process of sample pooling are shown in **Table 1**.

NOTE: Because pipetting errors may affect the accuracy of concentrations and protein quantitation, ensuring the pooling amount correctly is important. The discrepancies in intensity among 16 samples should be less than 5%.

3.4. Labeled peptide desalting

NOTE: Because the background derivatives in the TMT16 labeling reaction (e.g., TMTpro-NHOH from the hydroxylamine quenching reaction, and TMTpro-OH from TMT hydroxylation) are hydrophobic, an extensive wash condition is used for TMT16 labeled samples to effectively remove the derivatives. The addition of 5% ACN in the regular wash buffer (0.1% TFA) and 10x bed volumes of wash buffer are used.

3.4.1. Acidify the pooled samples by adding 10% TFA to pH < 3.

3.4.2. Centrifuge the pooled sample at 21,000 *x g* for 10 min and put the supernatant in a new tube.

3.4.3. Dry the sample using a vacuum concentrator to remove ACN.

3.4.4. Pre-condition a solid-phase extraction cartridge containing 50 mg sorbent column by washing the column with 2 mL of 100% methanol followed by 2 mL of elution buffer (60% ACN plus 0.1% TFA) and lastly 2 mL of wash buffer (0.1% TFA).

3.4.5. Load the sample on the column. Adjust the flow rate to ~100 µL/min to ensure the full extent of peptide binding. Save the flow through.

3.4.6. Wash the column three times with 1 mL wash buffer.

3.4.7. Elute peptides with 1 mL elution buffer.

3.4.8. Dry the eluted peptides in a vacuum concentrator and store the peptides at -80 ˚C for further fractionation.

4. Offline basic pH LC pre-fractionation

4.1. Fractionation system preparation

4.1.1. Prepare buffer A (10 mM ammonium formate, pH 8.0) and buffer B (10 mM ammonium formate, 90% ACN, pH 8.0) for a microliter flow high-performance LC system.

4.1.2. Setup an HPLC column containing bridged ethylene hybrid particles (3.5 µm particle size, 4.6 mm × 25 cm) in a microliter flow high-performance LC system for fractionation.

4.1.3. Install a 100 µL sample loop and wash the loop with 300 µL of methanol, water, and buffer A, sequentially.

4.1.4. Use 100 µL of 1:1:1:1 ratio of isopropanol : methanol : acetonitrile : water to wash the column. Then, further equilibrate the column for 1 h in 95% of buffer A.

4.2. Sample preparation

4.2.1. Dissolve the pooled and desalted TMT16 sample in 70 µL of buffer A. Confirm that the sample pH is ~ 8.0. If still acidic, then adjust the pH to 8.0 using 28% ammonium hydroxide (NH_4OH).

NOTE: To avoid sample loss, the sample volume should be less than 70% of the loop volume.

4.2.2. Centrifuge the sample at 21,000 *x g* for 10 min to remove precipitates.

4.3. Fractionation and concatenation

NOTE: Before real sample fractionation, a pilot experiment is highly recommended to ensure that the LC system is in good condition. This can be performed with a small amount of your actual sample (~5%) or with a non-TMT labeled mixture of peptides.

4.3.1. Inject the sample and fractionate it by the following gradient: 5% buffer B for 10 min, 5-15% buffer B for 2 min, 15–45% buffer B for 148 min and 45–95% buffer B for 5 min. Use a flow rate of 0.4 mL/min.

4.3.2. Set the fraction collector to collect fractions every 1 min and concatenate 160 fractions back to 40 fractions in 4 cycles.

NOTE: The concatenation is performed by combining early, middle, and late LC fractions eluted from the same time internals into a concatenated fraction. The concatenated fractions have

little overlap in the first dimension of LC thus increase the efficient usage of elution window in the second dimension LC. In addition, through several rounds of concatenation, the peptides can be evenly distributed across all the concatenated fractions. This approach has been demonstrated to increase the proteome coverage compared to the analysis of individual fractions[35,36].

4.3.3. Dry all concatenated fractions in a vacuum concentrator and store the dried samples at -80 ˚C for further LC-MS/MS analysis.

5. Acidic pH RPLC-MS/MS analysis

5.1. Acidic pH RPLC-MS/MS system preparation

5.1.1. Pack an empty column (75 µm ID with a 15-µm tip orifice) with 1.9 µm C18 resin to 10-15 cm in length.

5.1.2. Heat the column at 65 °C using a butterfly portfolio heater to reduce backpressure.

5.1.3. Wash the column thoroughly with 95% buffer B (3% dimethyl sulfoxide, 0.2% formic acid and 67% ACN). Then, fully equilibrate the column in 95% buffer A (3% dimethyl sulfoxide and 0.2% formic acid).

5.1.4. Check the performance of the LC-MS/MS system by running 100 ng of rat brain peptides or BSA peptides before analyzing the experimental samples.

5.2. LC-MS/MS analysis of concatenated fractions

5.2.1. Reconstitute the dried peptides from the basic pH fractions in 5% FA, and centrifuge at 21,000 × g for 5 min. Transfer the supernatant of each sample to an HPLC vial insert.

5.2.2. Load ~1 µg peptides of each fraction onto the column. The peptides are eluted at 0.25 µL/min flow rate with a 60 min gradient of 18–45% buffer B.

NOTE: To obtain high identification numbers, run one fraction and adjust the gradient for the remaining fractions based on the first run. The best gradient should have evenly distributed peptides across the entire gradient (**Figure 4A**).

5.2.3. Operate the mass spectrometer with the following parameters for the analysis of TMT16-labeled samples: MS1 scans (full MS scan range: 450-1600 *m/z*; Orbitrap resolution: 60,000; automatic gain control target: 1×10^6; maximum ion time: 50 ms) and 20 data-dependent MS2 scans (Orbitrap resolution: 60,000; AGC target: 1×10^5; maximum ion time: 110 ms; HCD normalized collision energy: 32%; isolation window: 1.0 *m/z*; isolation offset: 0.2 *m/z*; dynamic exclusion: 10 s).

NOTE: The parameters used here are optimized on one type of mass spectrometer (see **Table of Materials**). For different MS instruments, users should fine-tune instrument parameters to achieve high-quality results. One setting is to monitor normalized HCD collision energy, as the optimum energy may vary across instruments as well as between TMT11 and TMT16.

6. Data processing

NOTE: The data analysis was performed using a JUMP software suite[37-39] including a hybrid database search engine (pattern- and tag-based), filtering software that controls for the false-discovery rate (FDR) of identified peptides/proteins, and quantification software for TMT datasets. Depending on a user's situation, data analysis can be done using other commercial or freely available programs.

6.1. Database search

6.1.1. Convert the .raw files from the MS instrument to .mzXML files, and search MS2 spectra against a non-redundant target-decoy database[40] generated from UniProt human protein sequences (or another appropriate species-specific database) to calculate the FDR of identified proteins.

NOTE: Generate the non-redundant database by combining protein sequences from Swiss-Prot and TrEMBL databases. One can also add customized protein sequences not contained in those reference databases, including protease cleaved proteins, proteins with single nucleotide polymorphisms and common contaminants.

6.1.2. Conduct searches using the following parameters. Mass tolerance for precursor: 10 ppm; mass tolerance for product ions: 15 ppm; maximal missed cleavages: 2; maximal modification sites: 3; static modifications: 304.20715 Da for TMT16 tags on Lys residues and N termini, 57.02146 Da for carbamidomethylation on Cys residues; dynamic modification: 15.99492 Da for oxidation on Met.

6.2. Filter the search results

6.2.1. Filter the resulting peptide-spectrum matches (PSMs) by peptide length (>6 amino acids), mass accuracy of precursor ion, and JUMP-based matching scores (Jscore and ΔJn). Peptides are then grouped by peptide length, tryptic ends, modifications, missed cleavage sites, and charge state.

6.2.2. Filter the data further with the matching scores to achieve an FDR below 1% at either the protein (whole-proteome analysis) or peptide (phosphoproteome analysis) level.

NOTE: If positive-control peptides/proteins are missing at the filtering steps, then FDR may be increased to a reasonable level so that those peptides/proteins can be rescued.

6.2.3. For the peptides shared by more than one member of a protein family, cluster the matched members into one group.

NOTE: With the rule of parsimony, the group is represented by the homologous protein with the highest number of shared peptides and other proteins matched by unique peptides.

6.3. Protein quantification

6.3.1. Quantify proteins using a built-in program of a statistical software suite to summarize TMT reporter ion intensities over all matched PSMs.

6.3.2. Extract TMT reporter ion intensities from each accepted PSM and correct the raw intensities according to isotopic distribution of each labeling reagents (e.g., TMT16-126 generates 92.6%, 7.2%, and 0.2% of 126, 127C, and 128C m/z ions, respectively) and filter out low-intensity and/or highly noisy PSMs on the basis of user-defined thresholds. Normalize the quantitation data using trimmed-mean (or median) intensities of samples to correct loading-bias.

6.3.3. For each identified protein, calculate the mean-centered intensities across samples (i.e., relative intensities) of matched PSMs and summarize the relative intensities of the PSMs by taking sample-wise average. Convert the relative signals to absolute ones by multiplying the overall-average intensity of the three most abundant matched PSMs.

6.3.4. Correct quantification interference using a previously reported y1 ion correction approach[37] which assumes that the y1 ion intensity is correlated to the reporter ion intensity. By estimating the linear relationship between y1 and reporter ion intensities from clean scans, the interference level from the contaminated y1 ion intensity in noisy scans is derived and corrected.

NOTE: For TMT-labeled tryptic peptides, K-TMT and R residues are two representative y1 ions (376.27574 Da and 175.11895 Da, respectively) in an MS2 spectrum. If only one y1 ion is detected and is consistent with the identified peptide, then the MS2 is considered to be a clean scan. If both y1 ions are detected, then the MS2 is considered to be a noisy scan.

6.3.5. Transfer the protein quantitation values to a spreadsheet for further analysis. Use unsupervised data analysis methods such as PCA or clustering analysis to explore the distribution of samples. To identify differentially expressed proteins, use statistical methods such as *t*-testing and analysis of variance (ANOVA).

7. MS data validation

NOTE: Prior to performing time-consuming biological experiments, use at least one method of validation to evaluate the quality of MS data.

7.1. Manually inspect the MS/MS spectra of proteins of interest to validate the peptide sequence and TMT reporter ion intensities.

7.2. Use antibody-based approaches (e.g., western blotting or immunohistochemistry analysis) to verify changes in protein levels. To confirm the presence of native peptides, use synthetic peptides as internal standards. The peptides' MS/MS spectra and retention time during LC-MS/MS should be identical under the same conditions.

7.3. Use a targeted MS approach to verify protein changes.

REPRESENTATIVE RESULTS:

The protocol for the newly developed TMT16, including labeling reaction, desalting, and LC-MS conditions, has been systematically optimized[41]. Furthermore, we directly compared the 11-plex and 16-plex methods by using them to analyze the same human AD samples[41]. After optimization of the key parameters for TMT16, both TMT11 and TMT16 methods yield similar proteome coverage, identification, and quantification > 100,000 peptides in > 10,000 human proteins.

Because the TMT16 reagents are more hydrophobic than TMT11 reagents, TMT16-labeled peptides are likely to be more hydrophobic than TMT11-labeled peptides, which may account for different retention time (RT) in RPLC. Thus, we evaluated the impact of TMT16 on peptide RT compared with TMT11 by analyzing the TMT11- and TMT16-labeled peptide mixture using LC-MS/MS. We found that TMT16 has a significant influence on RT to the peptides with medium hydrophobicity but has little effect on the peptides of extremely high or low hydrophobicity. Therefore, the similar starting and ending buffer B concentrations in the LC gradient can be used for different TMT-labeled peptides.

We then optimized the online RPLC gradient for TMT16-labeled sample. The gradient for TMT16 is very similar to that of TMT11. The percentage of starting and ending buffer B are the same (e.g., 18% to 45%). But we noticed that the number of identified peptides in TMT16 dropped quickly at around 40% buffer B when using the same gradient that is used for TMT11. Thus, we slightly reduced the time of the gradient between 40% and 45%. We also made minor adjustments to this gradient for different fractions and different samples. After the gradient optimization, the identified peptides were evenly distributed throughout the gradient (**Figure 4A**).

To maximize the number of proteins identified and accurately quantified using the TMT16 method, we optimized the normalized collision energy (NCE) for the TMT16-labeled samples in our previous report[41]. Different NCEs (from 20% to 40%) were tested on the mass spectrometer during LC-MS/MS runs. Balancing the number of protein identifications and the reporter ion intensity, an NCE of 30-32.5% was chosen as the optimal HCD collision energy to be used for TMT16-labeled samples.

Ratio compression caused by co-eluted interfering ions has been a limitation of the isobaric

labeling techniques for protein quantitation. A previously published study using TMT11 method show that ratio compression can be nearly eliminated by extensive LC pre-fractionation, optimized MS settings, and post-MS data correction strategies[37]. We used these strategies including pre-MS extensive fractionation (40 basic pH LC fractions), application of narrow isolation window (1 *m/z*) in the MS setting, and y1 ion correction in both TMT11 and TMT16 proteome analyses of the same samples. After examining the correlation curve of protein fold change between TMT11 and TMT16 datasets, we found the slope was very close to 1, indicating that the ratio compression in TMT16 was not visibly higher than that in TMT11 under our experimental condition[41]. The consistent results were reported that the ratio compression has no difference when multiplexing level was increased from 11 to 16[13,45]. Thus, previously published strategies can be used to alleviate ratio compression, thereby significantly improving quantitation accuracy[27,37,44,46].

Finally, we compared the number of PSMs, unique peptides and unique proteins quantified in TMT11- versus TMT16-labeled samples (**Figure 4B**). The results show that PSMs of both methods are comparable; however, the quantified proteins and peptides are slightly lower in TMT16 method, which is consistent with other reports[12,13]. Our results indicate that the improvements in the TMT16 process along with the use of optimized LC-MS parameters provide high-throughput, deep proteome profiling of biological samples.

FIGURE LEGENDS:

Figure 1: Structure of the 16-plex TMT reagent. (**A**) Structure of the 16-plex TMT reagent, labeling process, mass shift after labeling, and the mass of the reporter ion are shown. (**B**) Heavy isotope–labeled structures of the reporter ions of TMT16 reagents.

Figure 2: Workflow of proteome profiling by 16-plex TMT-LC/LC-MS/MS. Protein extracted from 16 biological tissue samples was digested and labeled with 16 different TMT tags. Samples from 16 channels are pooled equally, and the mixture is fractionated and concatenated into 40 fractions by offline basic pH reverse-phase liquid chromatography (RPLC). Each fraction is further analyzed by acidic RPLC coupled with high-resolution mass spectrometry. The MS/MS raw files were processed. The brain tissue picture is cited from Medium.com with some modifications.

Figure 3: Protein quality control. (**A**) Quantification of extracted protein from tissue on a short SDS gel with BSA as the standard. The standard curve plots the BSA concentration and Coomassie-stained protein band intensity used for quantification. (**B**) SDS gel used for protein quality assay.

Figure 4: Representative results. (**A**) Peptide distribution in acidic LC. The optimized gradient of buffer B after correction of dead volume is aligned in the same plot. (**B**) The histogram shows the number of quantified PSM, unique peptide, and unique protein in TMT11 and TMT16 methods.

Table 1: Representative data showing the process of sample pooling in step 3.3.

DISCUSSION:

An optimized protocol for TMT16-based deep proteome profiling has been implemented successfully in earlier publications[12,13,41]. With this current protocol, more than 10,000 unique proteins from up to 16 different samples can be routinely quantified in a single experiment with high precision.

To obtain high-quality results, it is important to pay attention to critical steps throughout the protocol. In addition to all the QC steps discussed in our previous article[27], we include additional essential steps specific for the TMT16 process. These steps are important in insuring a successful experiment. For example, TMT reaction derivatives (e.g., TMTpro-NHOH from hydroxylamine quenching reaction and TMTpro-OH from TMT hydroxylation) are detected as prominent singly charged ions before desalting by the LC-MS/MS analysis. It is critical to remove them during the desalting step. We have tested different desalting conditions and found that the addition of 5% ACN in regular wash buffer combined with 10 × bed volumes wash for three times effectively removed the derivatives[41]. In addition, TMT16 has an increased mass compared to TMT11, therefore the full scan range starts from a higher *m/z* (450 instead of 410) for TMT16-labeled samples. Moreover, as the optimal collision energy for a peptide depends on the mass-to-charge and charge state of the precursor ion[21], the peptides labeled with different chemical labeling tags may have different optimal collision energies. For TMT16, the collision energy 30-32.5% is optimal for TMT16, which is slightly lower than TMT11.

Isobaric labeling is a powerful technique that provides high multiplexing capability. Although other techniques such as SILAC (stable isotope labeling by amino acids in cell culture)[47] and label-free provide alternative strategies for quantitating proteins[48], they suffer from low throughput. TMT16 can quantitate proteins across 16 different biological samples in theory. However, it is much more common to use some of these channels as biological replicates, providing more statistical power and helping generate reliable data. Using replicates or even triplicates is very critical, especially in systems where the expected change in protein concentration is nominal. It is important to understand the biology of the system before designing the experiment to include the appropriate number of replicates. Certain biological systems are not ideally suited for some of the quality control steps in this protocol. The premix ratio test is not used when using immunoprecipitation samples for the protocol due the large percentage of proteins expected to change. In these cases, the results would get skewed with premix test. This is also true in cases where at least 1 of the 10 samples is expected to vary greatly in protein expression (empty vector, proteasome inhibition, etc.). It is also suggested to use a TMT channel as an “internal reference” that can then be used to combine multiple batches of TMT16 experiments[49].

This protocol can be used for high-throughput global proteome profiling of complex biological samples to study differentially expressed proteins and cell signaling pathways and to understand disease biology. In addition, with slight modifications to the protocol, it can be used to study post-translational modifications such as phosphorylation, ubiquitination, methylation, and acetylation. Taking an integrated approach combining exhaustive large-scale proteomic

analysis along with other -omics pipelines such as genomics, transcriptomics, and metabolomics can provide insights to broaden understanding of intricate biological systems[30,50].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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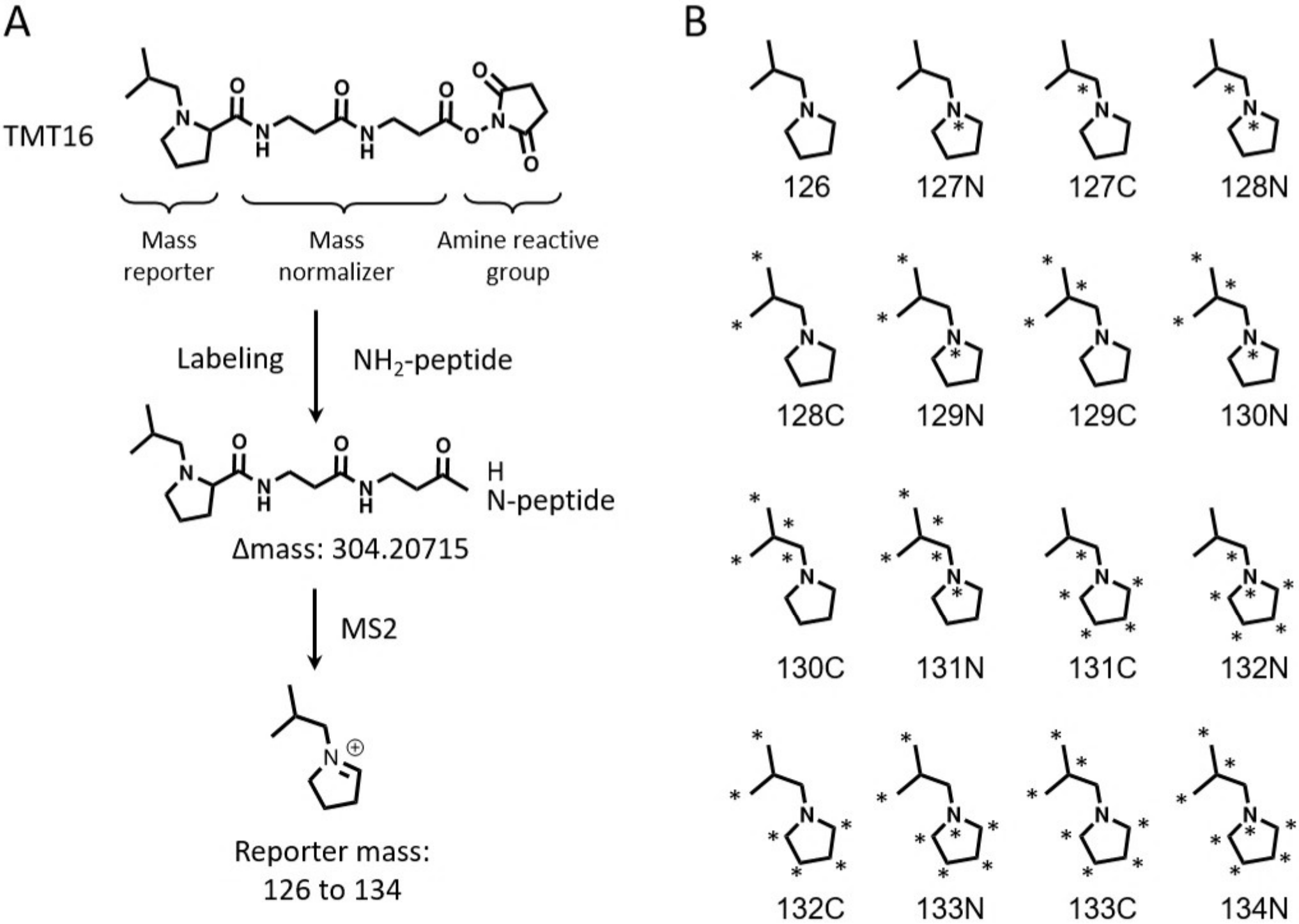

Figure

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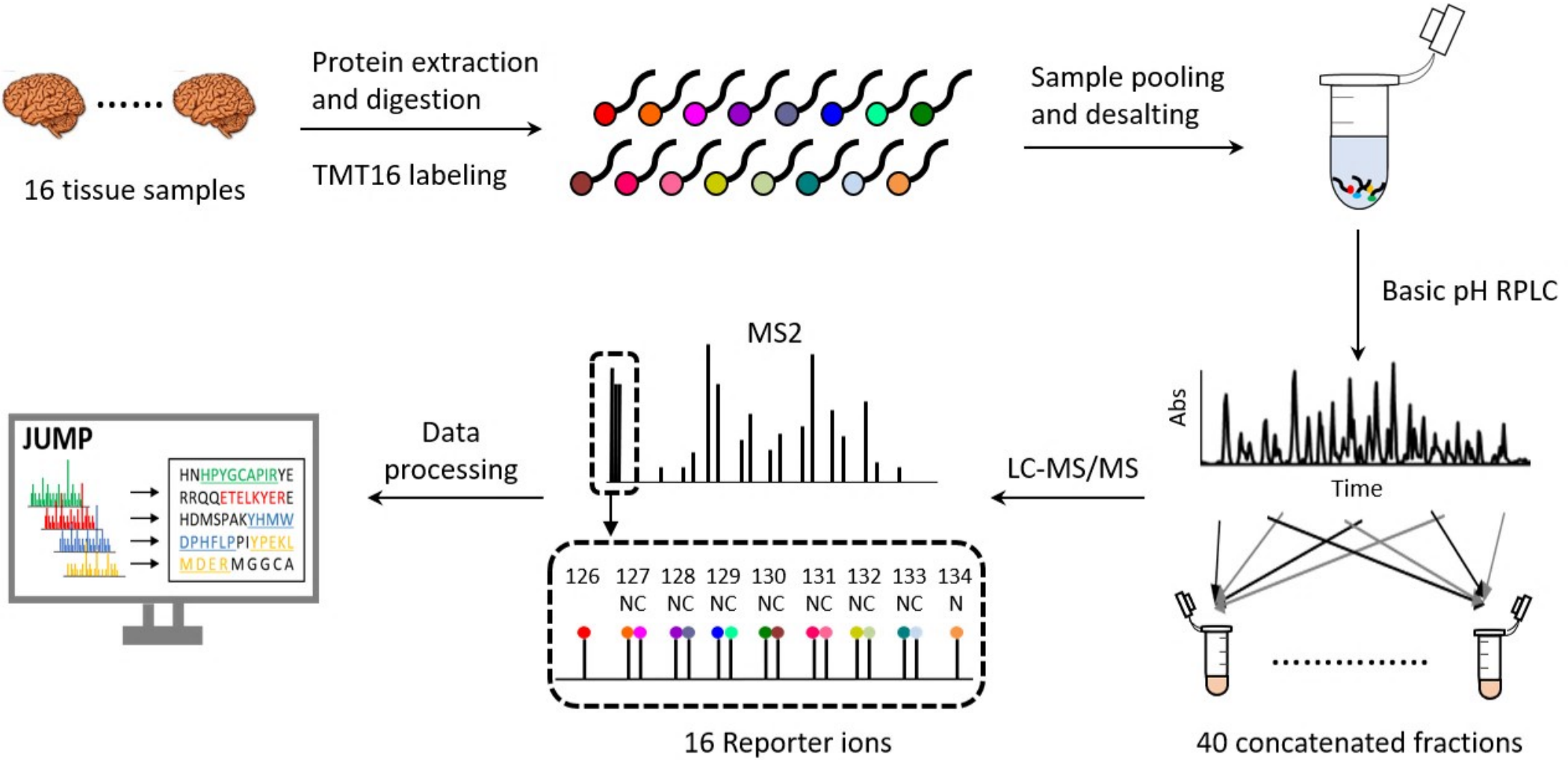

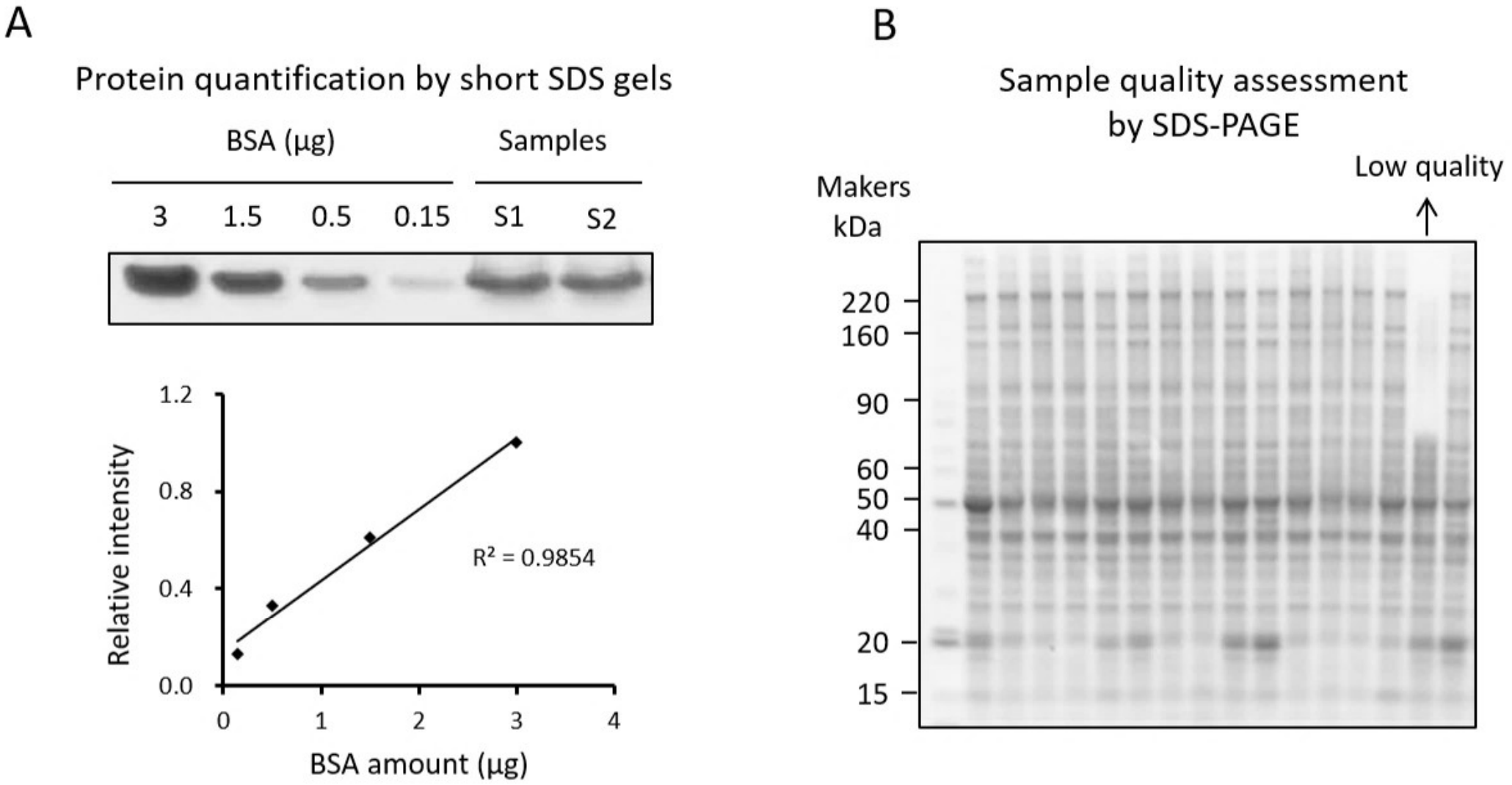

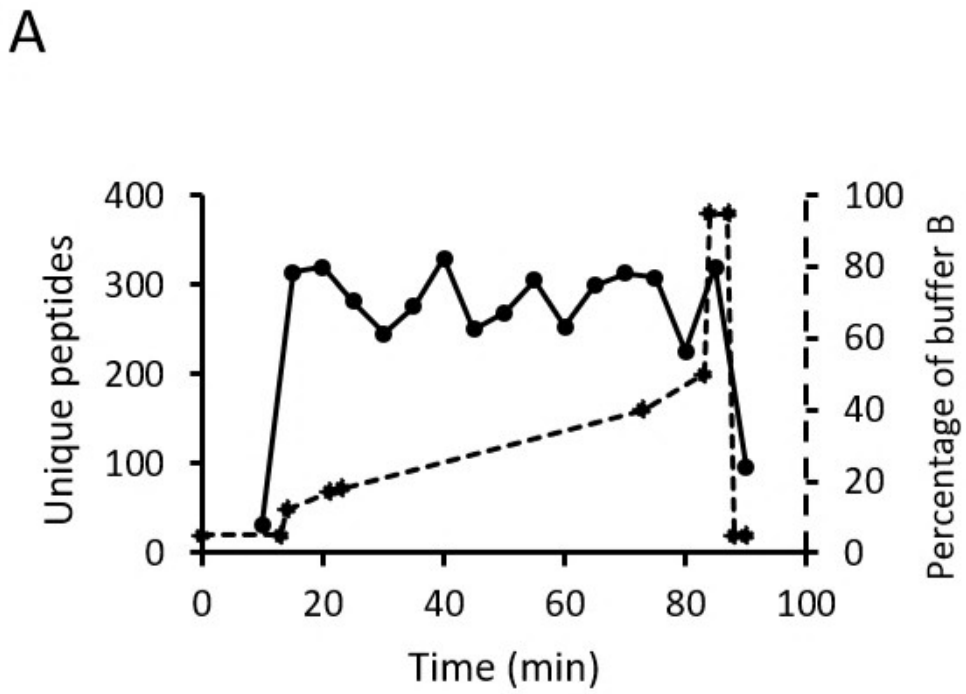

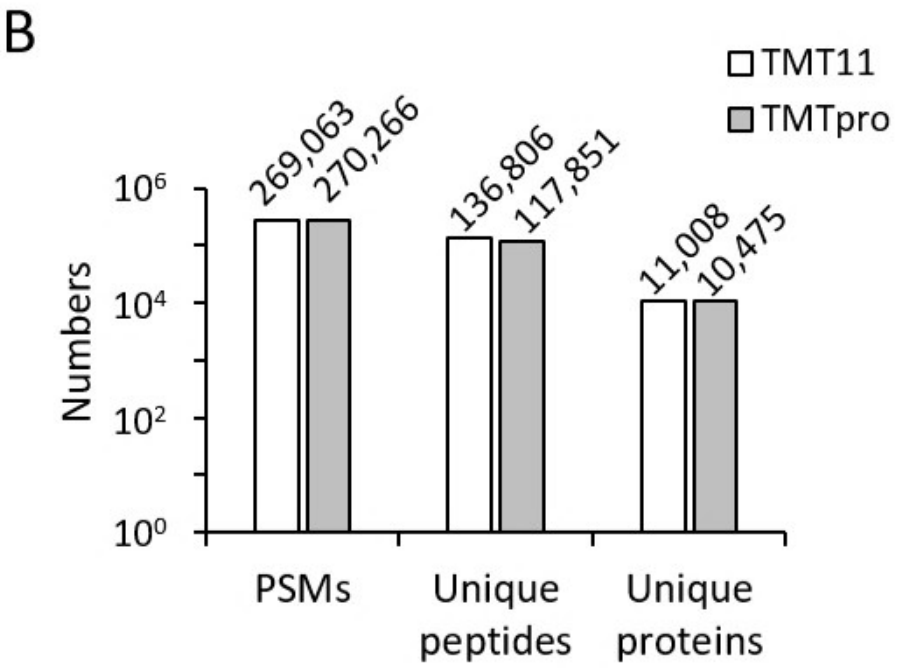

Table 1: A representative data showing the process of sample pooling in step3.3.

	1st (50 µl, use 50% in the first mix)				2nd (adjust the mixture and save 1		
Channels	Reporters	Mix Vol (µL	Intensity (u	Conc. (unit	Expected Ir	Added Vol	Total Vol (µ
1	sig126	25	94.7	3.8	122.1	7.2	32.2
2	sig127N	25	83	3.3	122.1	11.8	36.8
3	sig127C	25	86	3.4	122.1	10.5	35.5
4	sig128N	25	103.9	4.2	122.1	4.4	29.4
5	sig128C	25	90.8	3.6	122.1	8.6	33.6
6	sig129N	25	82.8	3.3	122.1	11.9	36.9
7	sig129C	25	101.3	4.1	122.1	5.1	30.1
8	sig130N	25	98.9	4	122.1	5.9	30.9
9	sig130C	25	86.3	3.5	122.1	10.4	35.4
10	sig131N	25	87	3.5	122.1	10.1	35.1
11	sig131C	25	119.1	4.8	122.1	0.6	25.6
12	sig132N	25	86	3.4	122.1	10.5	35.5
13	sig132C	25	119.1	4.8	122.1	0.6	25.6
14	sig133N	25	116.3	4.7	122.1	1.3	26.3
15	sig133C	25	122.1	4.9	122.1	0	25
16	sig134N	25	121.3	4.9	122.1	0.2	25.2

L0%)		3rd (final adjustment)			
Intensity (u	Conc. (unit	Expected Ir	Added Vol	Total Vol (µ	Intensity (units)
99.6	3.1	105.3	1.8	34.1	100
101.1	2.7	105.3	1.5	38.3	98
99.9	2.8	105.3	1.9	37.4	99.9
102.1	3.5	105.3	0.9	30.3	97.2
103.3	3.1	105.3	0.7	34.3	98.3
99	2.7	105.3	2.4	39.3	98.7
98.5	3.3	105.3	2.1	32.2	102.1
100.1	3.2	105.3	1.6	32.5	99.7
96	2.7	105.3	3.4	38.8	99.3
95.3	2.7	105.3	3.7	38.8	101.5
100.9	3.9	105.3	1.1	26.7	100.2
95.3	2.7	105.3	3.7	39.2	99.6
101.2	3.9	105.3	1	26.7	100
99.9	3.8	105.3	1.4	27.7	100.9
101	4	105.3	1.1	26.1	101.9
105.3	4.2	105.3	0	25.2	101.3

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
10% Criterion TGX Precast Midi Protein Gel	Biorad	5671035	
10X TGS (Tris/Glycine/SDS) Buffer	BioRad	161-0772	
4–20% Criterion TGX Precast Midi Protein Gel	Biorad	5671095	
50% Hydroxylamine	Thermo Scientific	90115	
6 X SDS Sample Loading Buffer	Boston Bioproducts Inc	BP-111R	
Ammonium Formate (NH4COOH)	Sigma	70221-25G-F	
Ammonium Hydroxide, 28%	Sigma	338818-100ml	
Bullet Blender	Next Advance	BB24-AU	
Butterfly Portfolio Heater	Phoenix S&T	PST-BPH-20	
C18 Ziptips	Harvard Apparatus	74-4607	Used for desalting
Dithiothreitol (DTT)	Sigma	D5545	
DMSO	Sigma	41648	
Formic Acid	Sigma	94318	
Fraction Collector	Gilson	FC203B	
Gel Code Blue Stain Reagent	Thermo	24592	
Glass Beads	Next Advance	GB05	
HEPES	Sigma	H3375	
HPLC Grade Acetonitrile	Burdick & Jackson	AH015-4	
HPLC Grade Water	Burdick & Jackson	AH365-4	
Iodoacetamide (IAA)	Sigma	I6125	
Lys-C	Wako	125-05061	
Mass Spectrometer	Thermo Scientific	Q Exactive HF	
MassPrep BSA Digestion Standard	Waters	186002329	
Methanol	Burdick & Jackson	AH230-4	
Nanoflow UPLC	Thermo Scientific	Ultimate 3000	
Pierce BCA Protein Assay kit	Thermo Scientific	23225	
ReproSil-Pur C18 resin, 1.9um	Dr. Maisch GmbH	r119.aq.0003	
Self-Pack Columns	New Objective	PF360-75-15-N-5	
SepPak 1cc 50mg	Waters	WAT054960	Used for desalting
Sodium Deoxycholate	Sigma	30970	
Speedvac	Thermo Scientific	SPD11V	
TMTpro 16plex Label Reagent Set	Thermo Scientific	A44520	

Trifluoroacetic Acid (TFA)	Applied Biosystems	400003	
Trypsin	Promega	V511C	
Ultra-micro Spin Column,C18	Harvard apparatus	74-7206	Used for desalting
Urea	Sigma	U5378	
Xbridge Column C18 column	Waters	186003943	Used for basic pH LC

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July 9, 2020

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Title: "High-throughput and Deep-proteome Profiling by 16-plex Tandem Mass Tag Labeling Coupled with Two-dimensional Chromatography and Mass Spectrometry"

Dear Dr. Vineeta Bajaj,

Thank you very much for reviewing our submission. We appreciate the comments from you as our editor and the four peer reviewers, as well as your suggestions during the revision. We have made revisions to strengthen the manuscript based on all the reviewers' comments.

The point-by-point response is attached below.

Thank you very much for handling our submission.

Sincerely,

Andy High
Junmin Peng

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you for the reminder, we have carefully proofread and edited the manuscript to avoid spelling and grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have formatted the manuscript according to the requirement.

3. Please provide an email address for each author.

The email addresses for all authors were added as suggested.

4. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …”

We have changed the summary to meet the requirement.

5. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

We have revised the abstract to meet the requirement.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Eppendorf, Bullet Blender, TMTpro, Ultra Micro Spin C18, C18 ZipTips, Q Exactive HF, JUMP software suite, Orbitrap, etc.

We have tried to avoid commercial words in the manuscript. For the mass spectrometer, it is important to mention the type of instrument we used because the parameter settings are different between instrument types.

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please remove the words TMTpro from the title and the text and use generic term instead. Please also remain neutral in tone and do not use the words like robust, simple, efficient, etc.

TMTpro has been replaced with a generic term (i.e. 16-plex TMT or TMT16).

We have revised the text to make it more neutral in tone.

8. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 95-96, 345-346, 403-407, 476-483, Section 7 of the protocol, 511-513, 522-524, 525-528.

We have modified these sections and the whole text to avoid the overlap with the previously reported paper. We generated an iThenticate report that showed no significant overlap with any single source.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We have revised the protocol to make sure that all the text is written in the imperative tense as suggested.

10. Please revise the text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We have removed “we”, “you”, and “our” from our protocol.

11. The Protocol should contain only action items that direct the reader to do something.

We have tried to use only action items in the protocol.

12. Please ensure you answer the “how” question, i.e., how is the step performed?

We have revised the protocol to make sure that it directs the reader how to perform each step.

13. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We have checked all the steps and divided the steps containing more than 3 actions into 2 steps.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the filmable content as required.

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Thank you for the reminder. We did not use any previously published figures.

16. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have removed all the figures from the manuscript. The figure title and the description are retained after the Representative Results section in the manuscript.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have revised the discussion section accordingly.

18. Please upload each figure individually to your editorial manager account.

We have removed the figures from the manuscript and uploaded them separately.

19. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

The symbols have been removed from the text.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript described an optimized method for 16-plex isobaric tandem mass tag (TMT) based large-scale proteomics. The manuscript is well prepared with each step well illustrated including tissue sample pretreatment, TMT labeling, LC-MS analysis and data processing. The protocol can potentially contribute to other researchers with a handful TMTpro method. However, there are a few minor problems needs to be addressed before the acceptance.

We appreciate the reviewer's positive comments.

Minor Concerns:
1. The TMT 16-plex can be named as TMTpro or TMTpro16-plex, not TMT16.

Thank you for pointing this out. Editorial comments have instructed us to avoid any commercial words therefore we still use TMT16 in our test, but we state that the TMT16 refers to 16-plex TMT in the main text.

2. Can the label efficiency step (section 3.2) and label ratio check step (section 3.3) be combined into one LC-MS run? How essential is a control run for the label efficiency check step? Will filtering out peptides with TMT labels and comparing with the total work?

Thank you for raising this important point. The labeling efficiency and sample pooling step cannot be combined into one LC-MS run. The control step is necessary to perform as it is important to ensure that all the samples are completely labeled before they are pooled together. In cases where samples are incompletely labeled they can be re-labeled with TMT reagents during the labeling efficiency step but not at the pooling stage.

3. Does lysC work well in 8M Urea? How was the digestion efficiency?

Yes, LysC works well in 8M Urea. We have performed several preliminary experiments to demonstrate that digestion efficiency was nearly 100%.

4. DMSO is bad for LC, are the rotor seals or piston seals wear out with DMSO?

The reviewer is correct that DMSO is bad for LC and that the rotor seals and piston seals wear out with DMSO overtime. We overcome this problem by routine maintenance of the LC systems and this has worked well for us. We have shown that the low percentage of DMSO in our buffer system increases the number of peptides we can identify.

Reviewer #2:
Manuscript Summary:
Well written and clear. A useful methods article.

We thank reviewer for the complimentary remarks.

Major Concerns:
None

Minor Concerns:
1. A few sections can be made more clear. In section 2.4.2 the desalting is done with an "ultra micro spin" column but in the table of materials this is listed as "C18ZipTips", which is confusing. Because there are multiple different desalting steps using different formats, it would be better to be explicit about which desalting device is being used (Millipore ZipTips, Waters SepPak, etc) at the different steps, and those should match the items in the table exactly.

The binding capacity of the desalting material must match to the peptide amount to be desalted. We have tested the binding capacities of different desalting products and chose the best material according to the amount of peptides to be desalted. For example, in the digesting and labeling efficiency test in section 2.3 and 3.2, we only take ~1 μg of peptide sample for desalting and further LC-MS analysis. We chose the C18 ZipTips, that performs well for up to 3 μg of peptides according to our test. In desalting total digested peptides (~50μg) in step 2.4, we used the ultra-micro spin column, which can desalt up to 250μg of peptides. To make the protocol more clear, we have added the description for the desalting materials in the material table.

2. In Figure 4B the TMT11 Unique peptides number label is mis-formatted

Thank you for the detailed review. It has been modified.

3. It would be nice to see a demonstration or representative data for step 3.3, pooling the samples and adjusting the mixing ratios to achieve a uniform mixture across all of the samples. I thought this section was a little unclear.

For the sample pooling, we mix half of each sample in the first round and analyze by LC-MS/MS. According to the mean intensity of each channel in the mixture, we adjust the mixing by adding the remaining samples into the mixture. We then repeat the adjustment until all samples are equally mixed (variation <5%, usually need 2-3 rounds of adjustment). To make the procedure more clear, we have added a table to show representative data for this step.

Table 1: A representative data showing the process of sample pooling.

	1st (50 μl, use 50% in the first mix)				2nd (adjust the mixture and save 10%)					3rd (final adjustment)			
Channels	Reporters	Mix Vol (μL)	Intensity (units)	Conc. (unit/μL)	Expected Intensity (units)	Added Vol (μL)	Total Vol (μL)	Intensity (units)	Conc. (unit/μL)	Expected Intensity (units)	Added Vol (μL)	Total Vol (μL)	Intensity (units)
1	sig126	25.0	94.7	3.8	122.1	7.2	32.2	99.6	3.1	105.3	1.8	34.1	100.0
2	sig127N	25.0	83.0	3.3	122.1	11.8	36.8	101.1	2.7	105.3	1.5	38.3	98.0
3	sig127C	25.0	86.0	3.4	122.1	10.5	35.5	99.9	2.8	105.3	1.9	37.4	99.9
4	sig128N	25.0	103.9	4.2	122.1	4.4	29.4	102.1	3.5	105.3	0.9	30.3	97.2
5	sig128C	25.0	90.8	3.6	122.1	8.6	33.6	103.3	3.1	105.3	0.7	34.3	98.3
6	sig129N	25.0	82.8	3.3	122.1	11.9	36.9	99.0	2.7	105.3	2.4	39.3	98.7
7	sig129C	25.0	101.3	4.1	122.1	5.1	30.1	98.5	3.3	105.3	2.1	32.2	102.1
8	sig130N	25.0	98.9	4.0	122.1	5.9	30.9	100.1	3.2	105.3	1.6	32.5	99.7
9	sig130C	25.0	86.3	3.5	122.1	10.4	35.4	96.0	2.7	105.3	3.4	38.8	99.3
10	sig131N	25.0	87.0	3.5	122.1	10.1	35.1	95.3	2.7	105.3	3.7	38.8	101.5
11	sig131C	25.0	119.1	4.8	122.1	0.6	25.6	100.9	3.9	105.3	1.1	26.7	100.2
12	sig132N	25.0	86.0	3.4	122.1	10.5	35.5	95.3	2.7	105.3	3.7	39.2	99.6
13	sig132C	25.0	119.1	4.8	122.1	0.6	25.6	101.2	3.9	105.3	1.0	26.7	100.0
14	sig133N	25.0	116.3	4.7	122.1	1.3	26.3	99.9	3.8	105.3	1.4	27.7	100.9
15	sig133C	25.0	122.1	4.9	122.1	0.0	25.0	101.0	4.0	105.3	1.1	26.1	101.9
16	sig134N	25.0	121.3	4.9	122.1	0.2	25.2	105.3	4.2	105.3	0.0	25.2	101.3

In step 5.2.1, is there a recommended volume or concentration to use for reconstituting the peptides immediately prior to analysis by LC/MS/MS?

Normally, we reconstitute peptides to a concentration of ~300 ng/uL and inject 2-3 uL sample for LC/MS/MS analysis.

Reviewer #3:
Manuscript Summary:

The manuscript by Zhen Wang and colleagues provides a fairly detailed protocol for 16-plex TMT proteomic analysis by MS/MS after 2-D reverse phase HPLC fractionation of samples. The method should be of interest to the proteomics community because the 2-D fractionation allows for very deep proteomic coverage (over 10,000 proteins), while reducing ratio compression which otherwise can be problematic for MS2 analysis, especially with 16-plex TMT. The method is compatible with widely available Q Exactive mass spectrometers, and does not require more expensive tribrid instrumentation as do other TMT workflows that attempt to address ratio compression. The novelty is minimal because the manuscript is a follow-up to a 2017 JoVE article by these authors, though this version introduces a few new tricks and specifically addresses a few issues specific to the TMT pro (16-plex) reagents. The beginning of the manuscript is clearly written, but the second half contains numerous grammatical mistakes that should be addressed. If several relatively minor issues are addressed, the article should be useful to many users of the relatively new 16-plex TMT reagents.

Major Concerns:
None

Minor Concerns:
1. Line 87- it should be mentioned that time of processing is especially important when analyzing labile posttranslational modifications, perhaps citing one of the early CPTAC papers that mention half-life of phosphorylation in tumor samples.

Thank you for the great suggestion. We have supplemented this point in the note.
"*To reduce the impact of sample harvesting on the proteome, it is crucial to collect samples in minimal time at low temperature if possible*[1,2]*. This is especially important when analyzing posttranslational modifications as they typically are labile, for example some phosphorylation events only have few seconds of half-life*[3,4]."

1. Eden, E.; Geva-Zatorsky, N.; Issaeva, I.; Cohen, A.; Dekel, E.; Danon, T.; Cohen, L.; Mayo, A.; Alon, U., Proteome Half-Life Dynamics in Living Human Cells. *Science* **2011,** *331* (6018), 764.
2. Hinkson, I. V.; Elias, J. E., The dynamic state of protein turnover: It's about time. *Trends in Cell Biology* **2011,** *21* (5), 293-303.
3. Mertins, P. *et al.* Ischemia in Tumors Induces Early and Sustained Phosphorylation Changes in Stress Kinase Pathways but Does Not Affect Global Protein Levels. *Molecular & Cellular Proteomics* 2014 **13** (7), 1690.
4. Kleiman, L. B., Maiwald, T., Conzelmann, H., Lauffenburger, D. A. & Sorger, P. K. Rapid phospho-turnover by receptor tyrosine kinases impacts downstream signaling and drug binding. Molecular Cell **2011,** 43 (5), 723-737,

2. Line 112: This is confusing. Authors should use w:w or v:v or something like "add 100 uL lysis buffer per 10 mg tissue". As is, 1:100 volume:weight is not correct, especially since 100uL of buffer would have a mass of about 100 mg assuming the same density as water for the lysis buffer.

We have changed this sentence to "add 100 uL lysis buffer per 10 mg tissue" as suggested.

3. line 172: I assume 50 uL is the final volume, this should be stated.

The reviewer is correct, we have added this statement.

4. line 250: Is the sample vortexed, or any other advice for resolubilization?

The peptides are easy to dissolve. We usually suspend the peptide in the solvent by vortexing several times. Ultrasonic dissolving is also suggested for resolubilization. We have added more details here about dissolving of peptides.
"*Resuspend each desalted peptide sample in 50 µL of 50 mM HEPES (pH 8.5) by vortexing several times or ultrasonic dissolving*."

5. line 285: What if the sample is not completely labeled? Should labeling be continued and if so, how? What is an acceptable level of labeling? Can incomplete labeling be corrected bioinformatically?

If labeling is not complete we add additional TMT reagents to label the remaining peptides and check the labelling efficiency again before quenching. In our analysis, we always make sure 100% labeling is achieved as unlabeled peptide will result in increased sample complexity which negatively affects the number of quantified peptides/proteins. In SILAC experiments, it is generally acceptable that the heavy amino acids be incorporated into >95% of the proteins for further quantitative experiment, as the quantification result can be represented from >95% of the specific peptides/proteins. This should be the case for chemical labeling strategies. Thus the near 100% labeling can be used for further bioinformatics analysis.

We have supplemented some steps for the situation that the sample is not completely labeled.
"*If the labeling is not completed, add additional TMT reagents to label the remaining peptides and check the label efficiency again before quenching.*"

6. line 342: presumably this is a sample loop? If so please state this.

The reviewer is correct, it is the sample loop. We have added the statement.

7. line 372: please elaborate on the concatenation approach or cite the original reference that describes this strategy in more detail (perhaps from the Gygi lab?).

We have added one note to explain the way to do the concatenation and its benefit.

"Note: The concatenation is performed by combining early, middle and late LC fractions eluted from the same time internals into a concatenated fraction. The concatenated fractions have little overlap in the first dimension of LC thus increase the efficient usage of elution window in the second dimension LC. In addition, through the several rounds of concatenation, the peptides can be evenly distributed across all the concatenated fractions. This approach has been demonstrated to increase the proteome coverage compared to that of an individual fraction[5,6]."

5. Wang, Y. et al. Reversed-phase chromatography with multiple fraction concatenation strategy for proteome profiling of human MCF10A cells. *Proteomics* **2011**. 11 (10), 2019-2026.
6. Yang, F., Shen, Y., Camp, D. G., 2nd & Smith, R. D. High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis. *Expert review of proteomics* **2012**. 9 (2), 129-134.

8. line 385: Why is 3% dimethyl sulfoxide added to the buffers?

According to the published reports and shown in our lab, the addition of DMSO to LC solvents enhances electrospray response and improves peptide/protein identification rates[7,8]. Thus the 3% DMSO is added in our LC solvents.

7. Hahne, H.; Pachl, F.; Ruprecht, B.; Maier, S. K.; Klaeger, S.; Helm, D.; Médard, G.; Wilm, M.; Lemeer, S.; Kuster, B., DMSO enhances electrospray response, boosting sensitivity of proteomic experiments. *Nature Methods* **2013**, 10 (10), 989-991.
8. Meyer, J. G.; A. Komives, E., Charge State Coalescence During Electrospray Ionization Improves Peptide Identification by Tandem Mass Spectrometry. *Journal of the American Society for Mass Spectrometry* **2012**, 23 (8), 1390-1399.

9. line 388: Why rat brain peptides?

The rat brain peptides are generated by digesting rat brain tissue sample in our lab for daily assessment of LC-MS/MS proteomic platforms. It is a complex sample, containing a broad range of peptide hydrophobicity with different retention times and MS responses that is ideal to assess LC-MS performance and suitability. After LC-MS/MS analysis of complex rat brain peptides, many crucial parameters, such as chromatographic peak shape, retention time shift, MS intensity and mass accuracy can be monitored over time.

10. line 413: Which reagent requires higher collision energy, or can this not be generalized?

According to our experimental results, TMT11-labeled peptides require slightly higher HCD collision energy than TMT16-labeled peptides (35% for TMT11, 32% for TMT16). As the optimal collision energy for a peptide depends on the mass-to-charge (*m/z*) and charge state of the precursor ion[9], the peptides labeled with different chemical labeling tags are expected to have different optimal collision energies. For the same chemical labeling, the optimal NCE should be similar when the samples are analyzed in the same instrument.

9. Kelstrup, C. D. et al. Rapid and Deep Proteomes by Faster Sequencing on a Benchtop Quadrupole Ultra-High-Field Orbitrap Mass Spectrometer. *Journal of Proteome Research* **2014**. 13 (12), 6187-6195.

11. line 435: Why allow up to 2 missed cleavages when tryptic digestion is closely monitored during the sample prep stage?

We monitor the tryptic digestion step to make sure we achieve high digestion efficiency, but missed cleavage sites are frequently observed in some sequences which have successive

lysine/arginine residues or acidic amino acids, aspartic and glutamic acids existing near the cleavage site. The setting of 2 maximal missed cleavages will help to identify these peptides.

12. line 547 and line 599: This is surprising, one would expect and I have read reports that there is more ratio suppression with TMT 16 reagents because the ratio of each individual channel to total sample is lower with 16-plex than 11-plex. Data should be shown or a paper cited if the authors want to claim that ratio compression is similar.

Ratio compression caused by co-eluted interfering ions have been a limitation of the isobaric labeling techniques for protein quantitation. Our previously published study using TMT11 methods show that ratio compression can be nearly eliminated by extensive LC pre-fractionation, optimized MS settings, and post-MS data correction strategy[10]. We used these strategies including pre-MS extensive fractionation (40 basic pH LC fractions), application of narrow isolation window (1 *m/z*) in the MS setting, and y1 ion correction in both TMT11 and TMT16 proteome analyses of the same samples. After examining the correlation curve of protein fold change between TMT11 and the TMT16 dataset, we found the slope was very close to 1, indicating that the ratio compression in TMT16 was not visibly higher than that in TMT11 under our experimental condition[11]. The consistent result was reported that the ratio compression has no difference when multiplexing level was increased from 11 to 16[12].
We have added more explanations to make it clearer.

10 Niu, M.; Cho, J. H.; Kodali, K.; Pagala, V.; High, A. A.; Wang, H.; Wu, Z.; Li, Y.; Bi, W.; Zhang, H.; Wang, X.; Zou, W.; Peng, J., Extensive Peptide Fractionation and y1 Ion-Based Interference Detection Method for Enabling Accurate Quantification by Isobaric Labeling and Mass Spectrometry. *Anal. Chem.* **2017,** *89* (5), 2956-2963.
11 Wang, Z.; Yu, K.; Tan, H.; Wu, Z.; Cho, J.-H.; Han, X.; Sun, H.; Beach, T. G.; Peng, J., 27-Plex Tandem Mass Tag Mass Spectrometry for Profiling Brain Proteome in Alzheimer's Disease. *Anal Chem* **2020,** *92* (10), 7162-7170.
12 Li, J.; Van Vranken, J. G.; Pontano Vaites, L.; Schweppe, D. K.; Huttlin, E. L.; Etienne, C.; Nandhikonda, P.; Viner, R.; Robitaille, A. M.; Thompson, A. H.; Kuhn, K.; Pike, I.; Bomgarden, R. D.; Rogers, J. C.; Gygi, S. P.; Paulo, J. A., TMTpro reagents: a set of isobaric labeling mass tags enables simultaneous proteome-wide measurements across 16 samples. *Nat. Methods* **2020,** *17* (4), 399-404.

13. Figure 4B: comma is misplaced in 136,806.

Thank you for the reminder, it have been corrected.

14. Line 573 should be "Proteins". There are other grammatical mistakes throughout, especially in the later parts of the manuscript.

Thank you for the detailed review. We have revised the whole text to avoid these mistakes.

15. line 575: state how many fractions are collected- I believe it is 160 here and 80 in the authors' previous JoVE article, if a range is allowed please state this.

The reviewer is correct that we collected 160 fractions and concatenated into 40 fractions. We have added more detailed statements to explain this.

We did not use the concatenation method in the previous JoVE article.

16. What about an empty channel to correct for isotopic impurities in the TMT labels?

We have performed the impurity test individually before we start the TMT labeling experiment. The impurity correction is conducted in our JUMP pipeline according to the TMT reporter impurity information.

Reviewer #4:
Manuscript Summary:
The authors present a very detailed protocol for the new 16-plex tandem mass tag reagents, TMTpro. The protocol covers the full experimental workflow from sample lysis to data analysis. Sufficient detail is given in each step from cell/tissue lysis though enzymatic digestion and labelling, to data analysis based on MS2 quantitation. The authors bring special attention the steps that are unique or deviate from the manufacture's recommendation. These include quality control checks after certain steps in the experiment that tend to be problematic. Enough detail is included throughout the protocol to allow this to serve as a reference for both the novice and seasoned researcher. This manuscript has a good balance between showing the power of the technique while also pointing out its limitations.

Major Concerns:
none

Minor Concerns:
As stated, the TMT11 identified ~5% more proteins and 16% more peptides than the TMTPro, but there were more PSMs observed from the TMTPro sample. Perhaps the authors can comment on this, if it is a chromatographic issue and more peptides are being re-sampled outside the dynamic exclusion window or something else other than chance?

During the LC-MS/MS analysis of TMTpro- and TMT11-labeled samples (40 fractions of each labeling), we analyzed one of the middle fractions first and adjusted the injection amount of other fractions according to the first run. During this process, we also monitored the MS signal and made some minor adjustments according to the previous run. When we finished all the runs, we found that we injected a higher number of peptides in some fractions for TMTpro analysis than TMT11. We speculate that this may be the reason that more PSMs were observed from the TMTPro sample.