Development of a Quality Control Standard for Tandem Mass Tags (TMT) Workflows

Jae Choi[1], Aaron M. Robitaille[2], Tabiwang N. Arrey[3], Rosa Viner[2], Andreas Huhmer[2], John C. Rogers[1]

[1] Thermo Fisher Scientific, 3747 N Meridian Rd, Rockford, IL 61101. [2] Thermo Fisher Scientific, 355 River Oaks Pkwy, San Jose, CA 95134. [3] Thermo Fisher Scientific Hanna-Kunath-Straße 11, 28199 Bremen, Germany

ABSTRACT

Quantitative proteomics strategies using Tandem Mass Tag™ (TMT™) reagents enable sample multiplexing and precise measurement of protein abundance. However, successful execution of this workflow includes multiple steps that may require optimization including chromatography, mass spectrometry (MS), and data analysis. Therefore to be able to detect and diagnosis co-isolation interference, enable MS method optimization and validation, we developed the Thermo Scientific™ Pierce™ TMT11plex labeled yeast peptide reference standard.

INTRODUCTION

Paulo et al. (JASMS, 2016) created a yeast triple gene knockout (TKO) TMT9plex standard to access ratio distortion using different instrument methods, as co-isolated ion interference can suppress accurate ratio quantification and thereby mask true differences in protein abundance across biological systems. To develop a widely applicable TMT11plex standard, we extended the above mentioned TKO sample by modifying the selection of yeast strains from *Paulo et al.* (JASMS, 2016) to select *met6Δ* and *ura2Δ* and *his4Δ*, as the original *pfk2Δ* line exhibited slower growth rates, and included the parental strain BY4742 as reference channels. Here we demonstrate how the TMT11plex labeled yeast peptide reference standard provides users a tool to measure the accuracy, precision and dynamic range of different mass spectrometry approaches, while also functioning as an excellent quality control assay to assess the LC and MS instrument status when combined with a standardized workflow.

MATERIALS AND METHODS

Four strains of *Saccharomyces cerevisiae* (a parental line and three lines respectively lacking the non-essential proteins Met6, His4, or Ura2) were used for the construction of the Pierce TMT11plex Labeled yeast peptide reference standard. Cultures were grown in YPD broth to an optical density (OD) of 3.0/mL and then harvested. Yeast cells were lysed via bead beating, and proteins lysates were then reduced, alkylated and digested with LysC and Trypsin. Samples were labeled with TMT11plex reagents according to manufacturer's instructions and mixed in equimolar ratios. Peptides from each knockout strain were labeled in triplicate, while the parental line was labeled in duplicate.

The samples were then analyzed on a range of Thermo Scientific™ Orbitrap™ mass spectrometers. All experiments were analyzed using a Thermo Scientific™ EASY-Spray™ C18 50cm column with a Thermo Scientific™ EASY-Spray™ ion source. Experiments run with a Thermo Scientific™ UltiMate™ 3000 RSLCnano UHPLC system used a gradient of 4-28% acetonitrile (vol/vol) gradient with 0.1% (vol/vol) formic acid in either 50min or 120min. Experiments run with a Thermo Scientific™ EASY-nLC™ 1200 HPLC system used a gradient with 8-32% acetonitrile (vol/vol) gradient with 0.1% (vol/vol) formic acid in either 50min or 120min. The Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap™ mass spectrometer was operated in data dependent mode with 120,000 MS1 / 45,000 MS2 resolution, 375-1500m/z, Top20, a 0.4m/z isolation window, 105ms MS2 injection time, NCE 34, and 20sec Dynamic Exclusion for 50min runs. The Thermo Scientific™ Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer was operated in data dependent mode with 120,000 MS1 / 45,000 MS2 resolution, 375-1500m/z, Top15, a 0.7m/z isolation window, 96ms MS2 injection time, NCE 34, and 20sec Dynamic Exclusion for 50min runs. For MS2 quantitation, the Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer was operated in data dependent mode with 120,000 MS1 / 50,000 MS2 resolution, 375-1500m/z, TopSpeed 2sec, a 0.7m/z isolation window, 105ms MS2 injection time, NCE 35, and 20sec Dynamic Exclusion for 50min runs. For SPS-MS3 quantitation, the Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer was operated in data dependent mode with 120,000 MS1 / IT-Turbo MS2 / 50,000 MS3 resolution, 375-1500m/z, TopSpeed 2sec, a 0.7m/z isolation window for MS2 and 2.0m/z for MS3, 50ms MS2 and 105ms MS3 injection time, 10 notches, MS2 NCE 35, MS3 NCE65, and 20sec Dynamic Exclusion for 50min runs. For MS2 quantitation, the Orbitrap Fusion Lumos Tribrid MS was operated in data dependent mode with 120,000 MS1 / 50,000 MS2 resolution, 375-1500m/z, TopSpeed 2sec, a 0.4m/z isolation window, 105ms MS2 injection time, NCE 35, and 20sec Dynamic Exclusion for 50min runs. For SPS-MS3 quantitation, the Orbitrap Fusion Tribrid MS was operated in data dependent mode with 120,000 MS1 / IT-Turbo MS2 / 50,000 MS3 resolution, 375-1500m/z, TopSpeed 2sec, a 0.4m/z isolation window for MS2 and 0.7m/z for MS3, 50ms MS2 and 105ms MS3 injection time, 10 notches, MS2 NCE 35, MS3 NCE65, and 20sec Dynamic Exclusion for 50min runs.

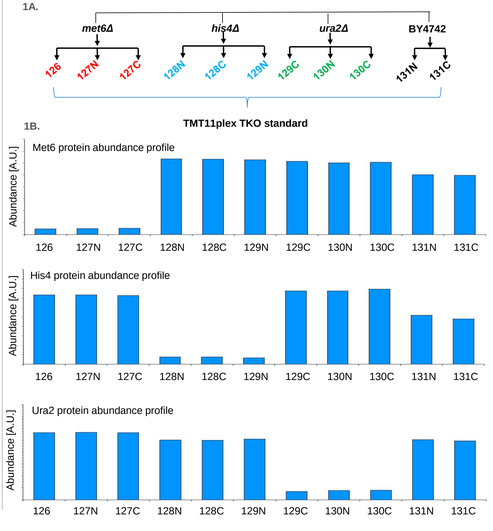
Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.2 or 2.3 software using SEQUEST® HT search with TMT6plex (229.163 Da) set as a dynamic modification, a 1% false discovery rate, 10ppm MS1 and 0.02 or 0.6Da MS2 mass tolerance, quantification of unique peptides with a co-isolation threshold of 50% for MS2 or 70% for SPS-MS3, and scaled to the paternal TMT11plex channels (131N and 131C). All reagents were purchased from Thermo Fisher Scientific.

RESULTS

We show that the Pierce TMT11plex labeled yeast peptide reference standard (Figure 1) can be used as a LC-MS system suitability standard to diagnose MS instrument status by monitoring mass accuracy, ion injection time, and reporter ion signal to noise (Figure 2 and 3) while also functioning as a method development tool to measure and optimize protein/peptides identification, acquisition, and data analysis methods. We demonstrate that the TMT11plex standard has less than 20% variability with lot to lot reproducibility (Figure 4). We then used the TMT11plex standard to establish a standardized workflow including two LC methods (50min for QC assay or 120min gradients for method development) for a variety of nano-spray liquid chromatography setups, optimized MS acquisition settings for hybrid or Tribrid Orbitrap mass spectrometers, and data analysis including statistics in Proteome Discoverer 2.2 software. As expected, synchronous precursor selection (SPS) based methods provided the best accuracy and precision as compared to MS2 methods. However, depending on precursor isolation purity and notches (fragments) selected for MS3, ratios for knock out proteins can be still distorted. To address the SPS selection issue, we implemented an additional filter into the Proteome Discoverer software quantitation node to exclude precursors if less than the user defined number of isolated fragments were from the identified peptide. Implementation of a new filter significantly improved quantitation accuracy and precision. Additionally, we benchmarked the proteomic standard using High pH fractionation, and evaluated the effect of modulating the advanced peak determination (APD) algorithm to optimize instrument methods (Figure 5).

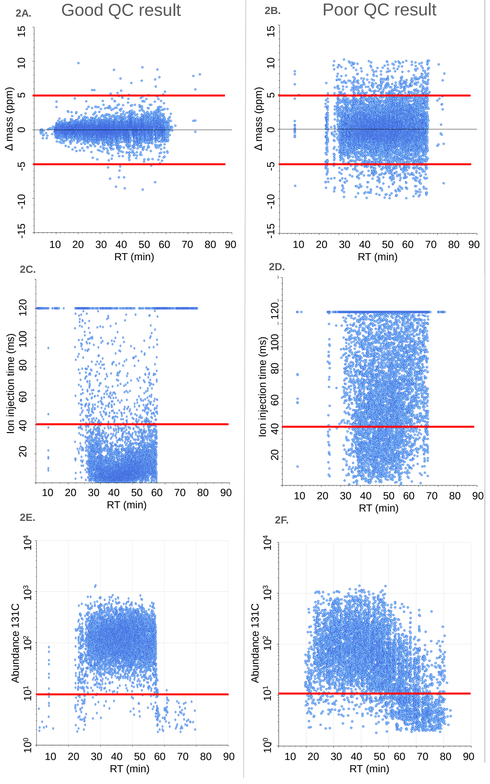
A TMT11PLEX LABELED YEAST PEPTIDE REFERENCE STANDARD

Figure 1. (1A.) Schematic representation of TMT11plex yeast triple gene knockout (TKO) standard. The standard is composed of four *Saccharomyces cerevisiae* strains: three lines respectively lacking the non-essential proteins Met6, His4, or Ura2, and the parental strain BY4742 for reference channels. (1B.) TMT reporter ions for Met6, His4, or Ura2 were measured using SPS-MS3 based method on the Orbitrap Fusion Tribrid mass spectrometer.



QUALITY CONTROL

Figure 2. To assess the readiness and health of the MS instrumentation, we employed the TMT11plex reference standard to monitored mass accuracy (2A-B), MS2 injection time as a readout for front optics health (2C-D), and reporter ion signal to noise as a readout for quadrupole cleanliness (2E-F). 500ng of the Pierce TMT11plex labeled yeast peptide reference standard was measured using a 50min gradient on a UltiMate 3000 RSLCnano system with a SPS-MS3 method on the Orbitrap Fusion Lumos Tribrid (Good QC) or Orbitrap Fusion Tribrid mass spectrometer (Poor QC). Red lines indicate suggested quality cut-offs.



PRECISION AND CONSISTENCY

Figure 3. We monitored the influence of MS instrumentation health on the quantification precision of a housekeeping protein Eno2. Protein abundance was scaled to the parental lines in Proteome Discover 2.2 and expected at 1:1 ratios. Data were collected as in Figure 2. Good QC (3A) and Poor QC (3B).

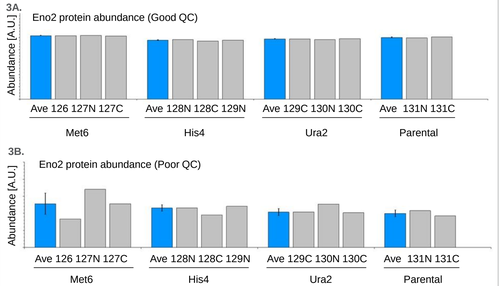
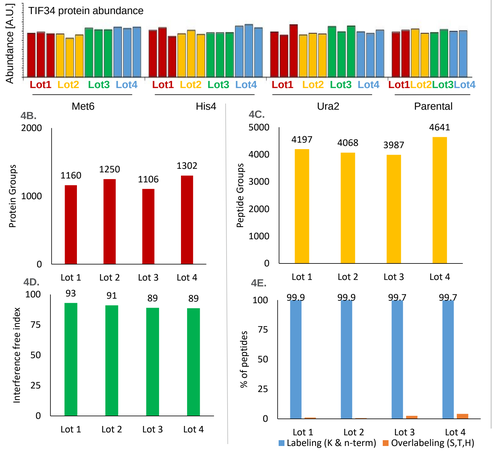
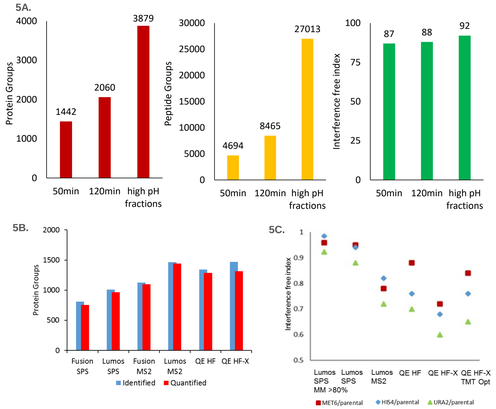


Figure 4. We monitored lot to lot reproducibility of the reference standard using a housekeeping protein TIF34 (4A), Protein IDs (4B), Peptide IDs (4C), Interference free index (4D), and TMT11 labeling efficiency (4E). Interference free index was calculated as average scaled protein abundance of parental - Met6, His4, or Ura2. 500ng of the reference standard was measured using a 50min gradient on a UltiMate 3000 RSLCnano system with a SPS-MS3 based method on the Orbitrap Fusion Tribrid mass spectrometer N=4.



OPTIMIZATION AND METHOD DEVELOPMENT

Figure 5. We benchmarked the reference standard using a 50min gradient, a 120min gradient, or eight High pH fractions analyzed for 120min per fraction and monitored protein ID, peptide ID, and interference free index (5A). Data shown here was collected on an Orbitrap Fusion Lumos Tribrid MS. We evaluated the TMT11plex reference standard on a range of Orbitrap instruments using a 50min gradient (5B). TMT Optimization mode (TMT Opt) on the Q Exactive HF-X MS or Post–acquisition filtering with a SPS Mass Match greater than 80% (MM >80%) in Proteome Discoverer 2.3 software improved the interference free index (5C).



CONCLUSIONS

We present a widely-applicable, high quality TMT11plex yeast peptide standard for LC-MS quality control and application development.

REFERENCES

1. Paulo, J.A., O'Connell, J.D., and Gygi, S.P. (2016) "A triple knockout (TKO) proteomics standard for diagnosing ion interference in isobaric labeling experiments," Journal of the American Society for Mass Spectrometry, 27(10) (pp. 1620–1625), doi: 10.1007/s13361-016-1434-9.

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TRADEMARKS/LICENSING

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