A TMTpro 18plex Proteomics Standard for Assessing Protein Measurement Accuracy and Precision

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ABSTRACT

Purpose: To generate a TMTpro-labeled yeast digest standard to measure the accuracy and precision of protein quantitation using different LC-MS methods and instruments.

Methods: Wild types, parental yeast strain(BY4741) was mixed with different fixed amounts of one or more knockout strains (Met6, His4, or Ura2). Samples were analyzed on an Thermo Scientific™ Eclipse Orbitrap™ and Exploris™ 480 mass spectrometers with or without a FAIMS Pro™ Interface. Raw data files were processed using Thermo Scientific™ Proteome Discoverer™ 2.5 and 3.0 software using the SEQUEST® HT and COMET search engines.

Results: Synchronous precursor selection (SPS)-based methods provided the best accuracy and precision compared to MS2 methods. The use of a FAIMS Pro Interface also improved the accuracy of the protein measurements for MS2 and MS3 methods. Overall, this new standard enables the assessment and optimization of instrument performance for TMTpro-labeled, multiplex samples.

INTRODUCTION

Multiplexed quantitation strategies using Thermo Scientific™ Tandem Mass Tags™ (TMT™) enable precise measurement of peptide or protein abundance from multiple samples using a single high-resolution LC-MS analysis. However, co-isolation of peptides with similar mass-to-charge can suppress protein abundance ratios resulting in less accurate measurements. Previously, we developed a multiplexed TMT11plex standard using three yeast knockout strains to assess co-isolation interference. Although this standard is useful for method optimization and assessing instrument performance, it does not enable accurate measurements of the knockout protein abundances. Here, we describe new prototype standards using TMTpro 18plex reagents capable of assessing both accuracy and precision of multiplex protein quantitation.

MATERIALS AND METHODS

Sample Preparation

Four strains of Saccharomyces cerevisiae (a parental line and three lines respectively lacking the non-essential proteins Met6, His4, or Ura2) used for the construction of the Pierce TMT11plex Labeled yeast peptide reference standard were also used for generation of the TMTpro 18plex 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. Standard curves were generated by mixing each knockout strain in fixed ratios before C18 SPE clean up. ~~Peptides from each knockout strain were labeled in triplicate, while the parental line was labeled in duplicate.~~

LC-MS Methods

The samples were ~~then~~ analyzed on a range of Thermo Scientific™ Orbitrap™ mass spectrometers. All experiments were performed 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. For MS2 quantitation, the Thermo Scientific™ Eclipse™ 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™ Eclipse™ 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 0.7 m/z for MS3, 50ms MS2 and 200 ms MS3 injection time, 10 notches, MS2 NCE 35, MS3 NCE65, and 20sec Dynamic Exclusion for 50min runs. For RTS quantitation, the Thermo Scientific™ Orbitrap™ Eclipse™ 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 0.7m/z for MS3, 50ms MS2 and 200ms MS3 injection time, 10 notches, MS2 NCE 35, MS3 NCE65, and 20sec Dynamic Exclusion for 50min or 60 ms for 120 min runs. Additional RTS parameters are shown in Figure 1.

Data Analysis

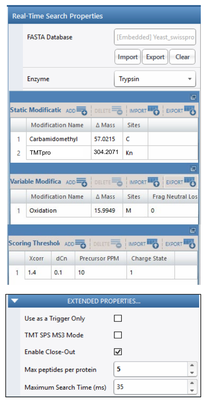
Data analysis was performed with Thermo Scientific™ Proteome Discoverer™ 2.5 or 3.0 software using SEQUEST® HT or COMET search engines with TMTpro (304.21 Da) set as a dynamic modification, a 1% false discovery rate, 10ppm MS1 and 0.02 or 0.6Da MS2 mass tolerance, quantification of unique peptides with a co-isolation threshold of 50% for MS2 or 70% for SPS-MS3 and scaled to the paternal strain channels (129N, 132N and 135N).

METHODS Cont.

Figure 1. Instrument acquisition methods for Thermo Scientific™ Orbitrap™ Eclipse™ mass spectrometer for RTS-MS3. Samples were acquired using different RTS parameters including trigger only, SPS-MS3, close out with 5 or 10 peptides selected. Raw data was analyzed using Proteome Discoverer™ 3.0 using COMET or SEQUEST database search engines.

MS Parameters

MS Parameter	
ICSW	3.5
MS1	
RF lens (%)	30
Resolution	120,000
AGC (%)	50
Ion Injection Time (ms)	25
Precursor Fit Filter (%, m/z)	50/1.2
Dynamic Ex (s)	60
MS2	
Isolation Window (m/z)	0.7
CID Collision Energy (%)	35
CID Activation Time (ms)	10
IT Scan Rate	Turbo
Scan Range Mode	Auto
AGC (%)	100
Ion Injection Time (ms)	35
MS3	
Isolation Window (m/z)	0.7
HCD Energy (%)	65
Resolution	50,000
Scan Range (m/z)	100-500
AGC (%)	250
Ion Injection Time (ms)	200



RESULTS

Figure 2. Schematic of TMTpro 18plex yeast digest standard prototype. Met6, His4, or Ura2 knockout yeast digests were mixed with parental yeast digest at fixed ratios 100%, 50%, 25%, 12.5%, 6.25% and 0% to generate samples with decreasing amounts of each protein. Each individual sample was labeled in triplicate using a distinct TMTpro 18plex tag. The combined sample results in a standard with a linear standard curves for each of the three knockout proteins that can be used to determine protein level abundance measurement accuracy and precision.

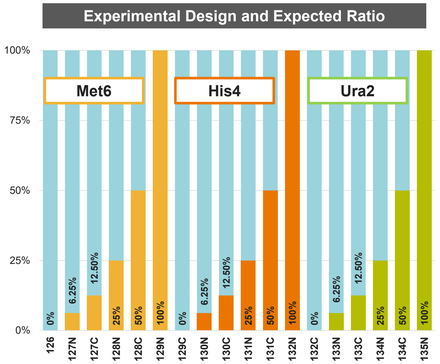
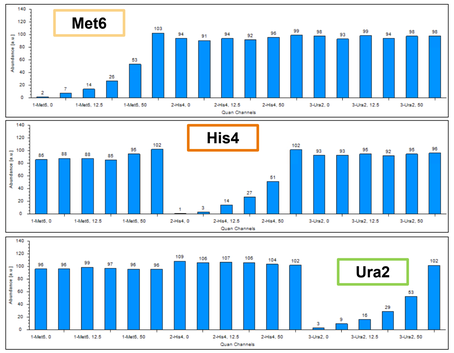


Figure 3. TMTpro 18plex Standard Reporter Ion Quantitation. Relative abundance of reporter ions from Met6, His4, or Ura2 proteins mixed with parental yeast digest at different fixed ratios. Data is from RTS-MS3 method using close out with 5 peptides used per protein. Comparison of protein measurement accuracy using different acquisition methods is shown in Figure 6.



RESULTS

Figure 4. Comparison of TMTpro 18plex yeast digest using different acquisition methods. Three different methods including high resolution (hr)MS2, SPS-MS3 and RST-MS3 (50 min gradient) were used to analyze the TMTpro 18plex yeast digest standard for MS/MS scans, PSMs, unique peptides and protein groups. The SPS-MS3 method resulted 400 fewer identified protein groups consistent with lower number of peptides, PSMs and MS/MS scans. The RTS-MS3 method provided the most protein group identifications due to more MS/MS scan and higher peptide identification ~~success~~ rates.

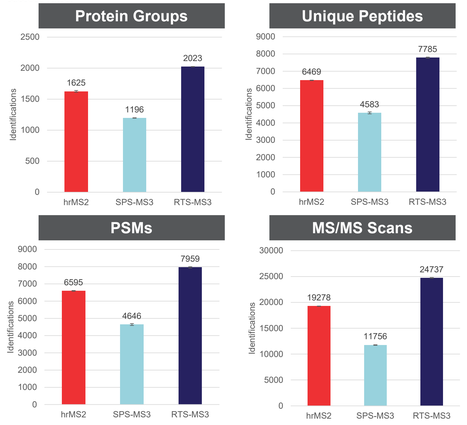


Figure 5. Protein quantitation improvements using RTS with COMET for database searching. Using RTS increases the total number of proteins identified compared to SPS-MS3 methods. More importantly, this mode resulted in a 26% increase in the number of quantifiable proteins. As RTS uses COMET for peptide sequencing during MS/MS acquisition, using the same search engine for peptide identification post acquisition increased the number of quantified proteins by 7% compared to using SEQUEST.

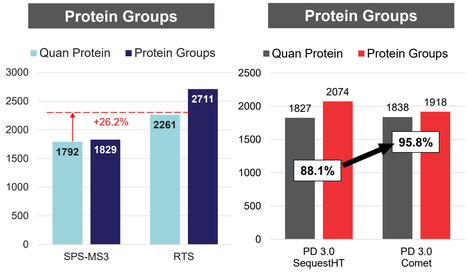
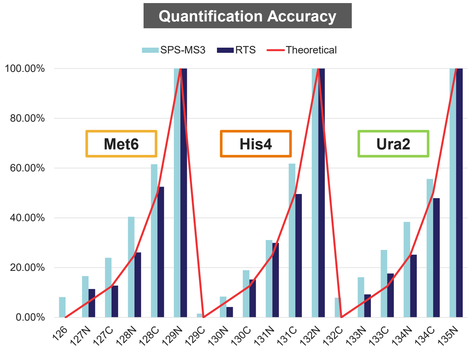


Figure 6. Improved quantification accuracy using RTS. Compared to the theoretical expected normalized protein quantitation for each sample (red line), the RTS method resulted in the more accurate measurement than SPS-MS3 alone. Accuracy improvements were observed for all three knockout proteins with Ura2 being the most accurate compared to the theoretical abundance level. Plotting these as a standard curve we observed a linearity of >0.98 for the first four serial dilutions but the linearity decreased to 0.9 when including the lowest concentration. In addition, RTS significantly reduced the background interference for the knockout only channels (126, 129C & 132C) with no signal observed compared to the original SPS-MS3 method.



CONCLUSIONS

- TMTpro 18plex Yeast Digest Accuracy Standard was designed by mixing knock-out different fixed ratios strains with a parental strain to generate standard curves to determine quantitative accuracy and precision.
- Orbitrap Eclipse Tribrid MS RTS method was able to increase the number of quantified proteins by 30% compared with SPS MS3.
- Orbitrap Eclipse Tribrid MS RTS and RTS close out methods enhanced the quantification accuracy of knock-out channels

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):1620–1625, doi: 10.1007/s13361-016-1434-9.

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