Increasing the Multiplexing of Protein Quantitation from 6- to 10-Plex with Reporter Ion Isotopologues

Rosa Viner,[1] Ryan Bomgarden,[2] Michael Blank,[1] John Rogers[2]
[1]Thermo Fisher Scientific, San Jose, CA, USA; [2]Thermo Fisher Scientific, Rockford, IL, USA

Thermo SCIENTIFIC

Overview

Purpose: To develop Tandem Mass Tag™ (TMT™) 10-plex reagents by combining current Thermo Scientific™ TMTsixplex™ reagents with four TMT6 reagent isotope variants on multiple high-resolution mass spectrometry platforms.

Methods: HeLa cell lysates and bovine serum albumin (BSA) digests were labeled with Thermo Scientific™ TMT10plex™ reagents. Aliquots from six, eight or all ten channels were mixed in different ratios and analyzed on three different Thermo Scientific™ Orbitrap™-based instruments.

Results: We have extended the multiplexing capabilities of TMT reagents from 6 to 10 without increasing the size or structure of the tag by utilizing the 6 mDa mass difference between ^{13}C and ^{15}N isotopes of the mass tag reporter ions. Mass spectrometry analysis on three different Orbitrap-based instruments using FT MS2/MS3 shows no negative effect on the number of protein identifications or quality of quantification in a complex digest by increasing sample multiplexing from 6 to 10.

Introduction

Amine-reactive isobaric tags (TMT or iTRAQ®) enable concurrent identification and multiplexed quantification of proteins in different samples using tandem mass spectrometry. Increasing the number of isobaric tag-labeled samples that can be compared in a single experiment is highly desirable. However, it has been reported that increasing multiplexing by using larger tagging molecules significantly decreases the number of identified and quantified proteins and peptides.[1] Here, we demonstrate that 10-plex multiplexing can be achieved with high resolution mass spectrometry utilizing the 6 mDa mass difference between ^{15}N and ^{13}C stable isotopes by combining current TMTsixplex reagents with four TMT6 isotope variants without loss of protein identifications and quality of quantification in a complex digest.

Methods

Sample Preparation

HeLa cell lysates (Thermo Scientific, Rockford, IL) and bovine serum albumin (BSA, Sigma, St.Louis, MO) were reduced, alkylated and digested with LysC and/or trypsin. Samples were then labeled with TMT10plex reagents (Figure 1) according to manufacturer's instructions. Aliquots from six, eight or all ten channels were mixed in equimolar ratios. To assess the impact of peptide co-isolation on quantification, HeLa digest labeled with Thermo Scientific™ TMT8plex™ reagents was spiked with BSA digest labeled with TMT10plex reagents mixed at fixed ratios (16:8:4:2:1:1:2:4:8:16).

LC-MS

A Thermo Scientific™ EASY-nLC 1000™ HPLC system and Thermo Scientific EASY-Spray™ source with Thermo Scientific™ Acclaim™ PepMap™100 2 cm x 75 µm trap column and Thermo Scientific™ EASY-Spray™ PepMap™ RSLC C18 25 or 50 cm x 75 µm ID column were used to separate peptides with a 5-25% acetonitrile gradient in 0.1% formic acid over 60 min for single proteins or 210 min at a flow rate of 300 nL/min for HeLa digest. Samples (500 ng injections) were analyzed on Thermo Scientific™ Q Exactive, Orbitrap Elite™ and Orbitrap Fusion™ Tribrid™ mass spectrometers using FT HCD MS2 or synchronous precursor selection (SPS) MS3 fragmentations (Orbitrap Fusion MS).

Data Analysis

Thermo Scientific™ Proteome Discoverer™ software version 1.4 was used to search MS/MS spectra against the Swiss-Prot® human+BSA database using SEQUEST HT® search engine. Static modifications included carbamidomethylation (C) and TMTsixplex (N-terminal, K). Dynamic modifications included methionine oxidation and deamidation (N,Q). Resulting peptide hits were filtered for maximum 1% FDR using the Percolator algorithm.[2] The TMT10plex quantification method within Proteome Discoverer software was used to calculate the reporter ratios with mass tolerance ±10 ppm without applying the isotopic correction factors. Only peptide spectra containing all reporter ions were designated as "quantifiable spectra". A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the peptides pertaining to that protein. For spiked in BSA experiments with data originating from the MS2 HCD method, a precursor co-isolation filter of 25% was applied. This eliminated peptides where contributions from co-eluting, nearly isobaric peptide species could interfere significantly with the reporter ion signals coming from the peptide of interest. For data sets obtained with the MS3-based method which contained both MS2 and MS3 spectra, CID(MS2) spectra were used for peptide identification and HCD(MS3) that contained reporter ions were used for quantification[3].

Results

Comparison of unlabeled to TMT6-, 8- or 10-plex labeled HeLa digests.

We have extended the previously reported[4,5] TMT8plex set of reagents to a 10plex set for multiplex peptide quantitation without increasing the size or altering the structure of the tag (Figure 1). The TMT10plex reagents utilize the 6 mDa mass difference between ^{13}C and ^{15}N isotopes which are measured by high-resolution LC-MS^n. In order to accurately quantify all ten channels, all reporter ions need to be baseline resolved. We determined the minimum resolution to separate all ten channels for an MSn resolution to be 35K at m/z 200 (~50K at m/z 120) as shown in Figure 2. At this resolution, an accurate ratio calculation for the all reporter ions can be achieved using a mass tolerance window of up to 10 ppm without applying isotope correction factors.

FIGURE 1. Thermo Scientific TMT10plex Reagents structures and reporter ion mass for TMT6plex tags (blue), TMT8plex (green) and TMT10plex (orange).

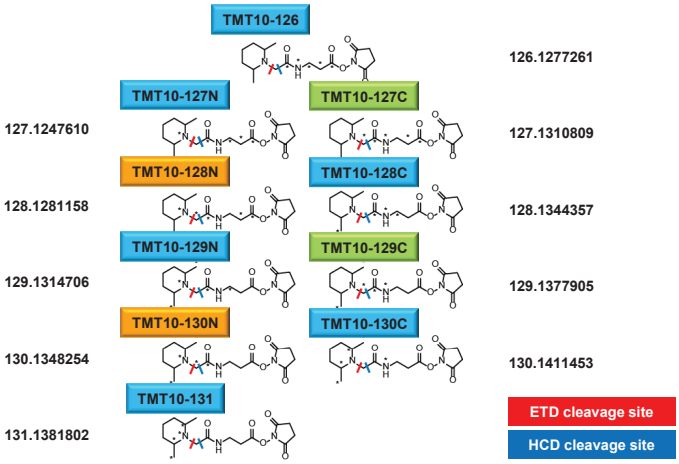
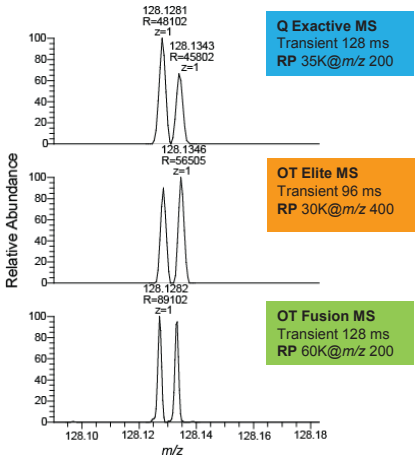


FIGURE 2. Optimal resolution settings to resolve isotope variants.

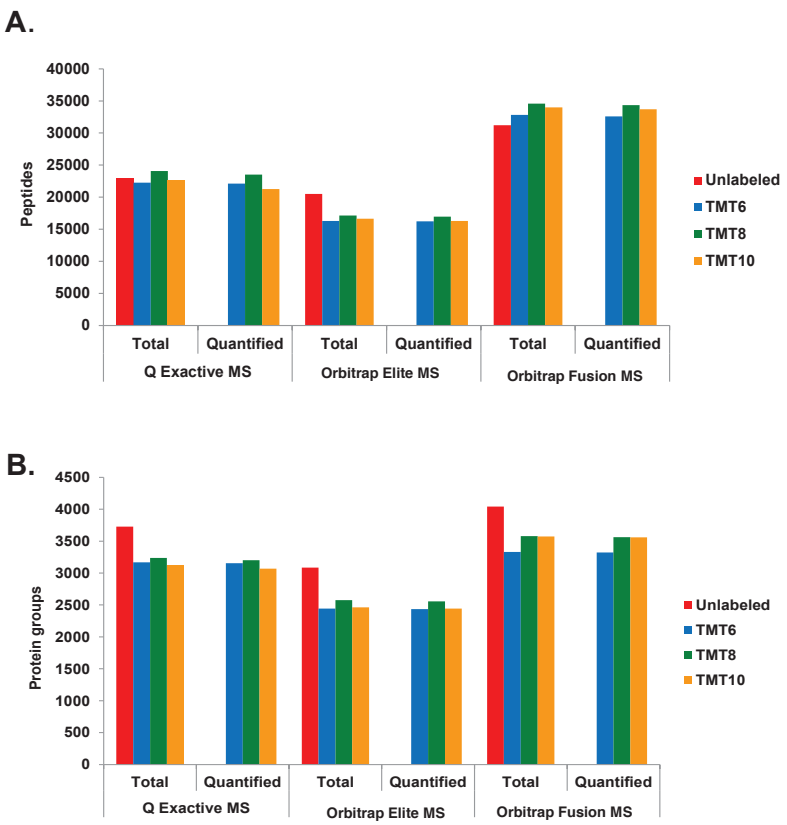


To benchmark the performance of new higher multiplexing reagents, we prepared a Lys-C, trypsin HeLa cell digest and analyzed unlabeled and TMT-labeled samples (equimolar 6-, 8- or 10-plex) on three different Orbitrap-based instruments. Acquisition methods used for Orbitrap Elite, Q Exactive and Orbitrap Fusion MS are summarized in Table 1. Compared to unlabeled HeLa digest, HCD MS2 of TMT6plex-labeled samples consistently identified at least 80% as many protein groups, with no loss in identified proteins/peptides with 8plex and 10plex samples compared to TMT6plex-labeled samples, depending on the instrument (Figure 3). All three multiplexed samples consistently quantified more than 98% of identified peptides and proteins compared to unlabeled samples.

TABLE 1. Instrument settings used for TMT experiments.

Parameter	Q Exactive MS	Orbitrap Elite MS	Orbitrap Fusion MS
FT MS1			
Resolution settings (FWHM at m/z 200)	140,000	144,000	120,000
Target value	3e6	1e6	2e5
Injection time	120	120	50
FT MS2	HCD	HCD	HCD
Resolution settings (FWHM at *m/z* 200)	35,000	36,000	60,000
Target value	1e5	5e4	1e5
Injection time	250	250	120
Isolation width (Da)	2	2	2
Top N	10	15	3 sec

FIGURE 3. Identification and quantification results for TMT experiments on three Orbitrap instruments. Number of total peptide identifications (A) and protein groups (B) are shown at 1% FDR for 500ng HeLa digest. The number of quantifiable proteins and peptides is also shown. Results represent an average of two replicate runs for each sample.



Quantitative precision and accuracy.

Two main factors contribute to decreased quantitative accuracy when using isobaric tags: 1) interference in reporter ion mass regions and 2) interference from co-isolation of precursor ions. Interference from background ions in the reporter region can be addressed by scanning with high resolution (Figure 2). Co-isolation interference has been recently addressed by using a multi-notch (SPS)MS3 experiment on an LTQ Orbitrap Velos or Orbitrap Elite instrument[4,6] and by combining a narrow precursor isolation width and fragmentation at the apex of the LC.[7]

FIGURE 4. Synchronous Precursor Selection MS3 TMT workflow for accurate quantification.

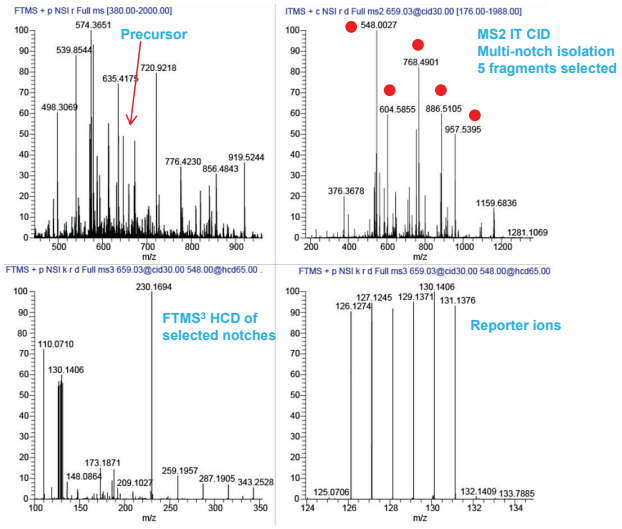


FIGURE 5. Accuracy and precision of TMT10 BSA quantitation spiked into TMT8 HeLa matrix using MS2 vs MS3 methods on Orbitrap Fusion MS. Expected (A) and observed (B) ratios normalized to 126 reporter ion intensity. Results represent an average of two replicate runs for each sample.

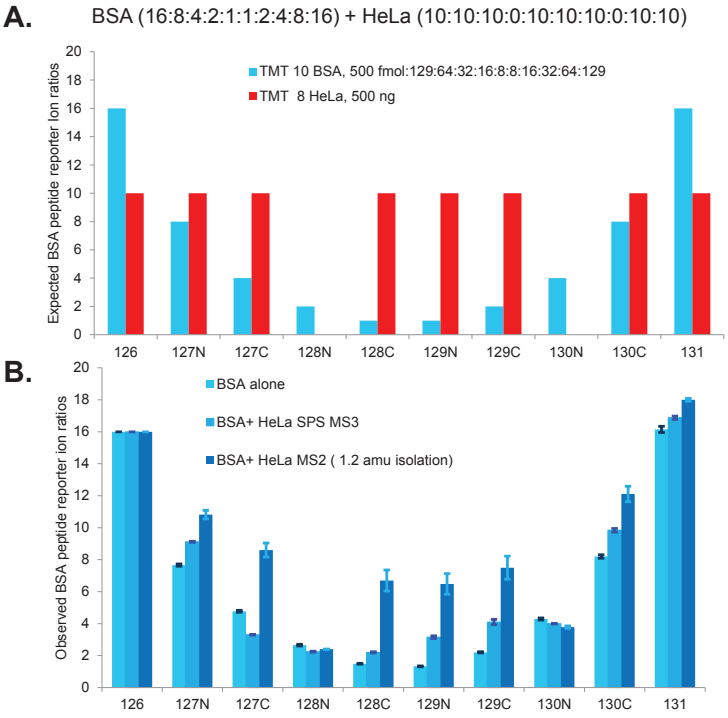
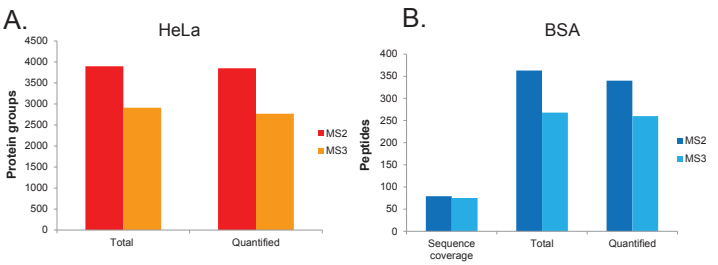


FIGURE 6. Identification and quantification of TMT10-labeled BSA digest spiked into TMT8-labeled HeLa using MS2 vs MS3 methods on the Orbitrap Fusion MS. Number of HeLa proteins (A) and BSA peptide (B) identifications are shown at 1% FDR. Results represent an average of two replicate runs for each sample.



To assess the impact of peptide co-isolation on quantification, TMT8plex-labeled HeLa samples were equally mixed (1:1:1:1:1:1:1:1) and spiked with BSA labeled with TMT10plex reagents mixed at different ratios (16:8:4:2:1:1:2:4:8:16). The sample (500 fmol BSA + 500 ng HeLa digest) was then analyzed on an Orbitrap Fusion MS using narrow precursor isolation (1.2 amu) by HCD MS2 or a novel SPS MS3 fragmentation/quantification method (Figures 4 & 5).[4] The SPS(multi-notch) MS3 method significantly improved quantitative accuracy for the spiked BSA peptide digest as shown in Figure 5B. The percent of proteins quantified employing the MS3 approach was over 95% with less than 25% loss in identified proteins due to the improved scan rate of the Orbitrap Fusion MS (Figure 6). These results significantly outperform previously reported single notch MS3 acquisition methods for both number of quantified proteins and quantitative accuracy.[3,6]

Conclusion

- Mass spectrometry analysis on three different Orbitrap-based instruments shows no negative effect on number of protein identifications or quality of quantification in complex digest by increasing TMT multiplexing from 6 to 10.
- Higher-resolution analysis, at least 35K@ m/z 200, is required for the accurate TMT10plex reagent reporter ion measurements.
- Precursor co-isolation effect in complex samples can be addressed to a large extent by relying on the reporter ion intensities extracted from MS3 spectra.
- The Orbitrap Fusion MS demonstrated significant improvements in the number of peptide identifications and quantifiable proteins using TMT reagents compared to the previous series of instruments.
- A novel FT MS3 Synchronous Precursor Selection method implemented on the Orbitrap Fusion MS showed only ~25% loss of identified proteins and an excellent 95% quantitation rate with significantly improved quantification accuracy for BSA digest peptides spiked into a complex proteome matrix.

References

1. Pichler, P.; Kocher, T.; Holzmann, J.; Mazanek, M.; Taus, T.; Ammerer, G.; Mechtler, K. Anal. Chem. 2010, 82: 6549−6558.
2. Kall, L. Canterbury, J, Weston, J., Noble, W.S., MacCoss, M. Nature Meth. 2007, 4: 923-925.
3. Viner, R.; Scigelova, M.; Zeller, M.; Oppermann, M.; Moehring, T.; Zabrouskov, V. Thermo Fisher Scientific Application Note #566, 2012
4. McAlister, G., Huttlin, E.L.; Haas, W.; Ting, L.; Jedrychowski, M.P.; Rogers, J.C.; Kuhn, K.; Pike, I.; Grothe, R.A,; Blethrow, J.D.; Gygi, S.P. Anal Chem. 2012. 84: 7469-7478.
5. Werner, T., Becher, I.; Sweetman,G.; Doce,C.; Savitski, M.; Bantscheff, M. Anal Chem. 2012. 84: 7188-7194.
6. Ting, L.; Rad, R.; Gygi, S. P.; Haas, W., Nature Methods 2011, 8 , 937-940.
7. Savitski, M. M.; Sweetman, G.; Askenazi, M.; Marto, J. A.; Lang, M.; Zinn, N.; Bantscheff, M. Anal. Chem. 2011, 83: 8959−8967.

www.thermoscientific.com

Certified System ISO 9001 QMI-SAI Global

Thermo Fisher Scientific, San Jose, CA USA is ISO 9001:2008 Certified.

Africa-Other +27 11 570 1840
Australia +61 3 9757 4300
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland/Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
Italy +39 02 950 591
Japan +81 45 453 9100
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Russia/CIS +43 1 333 50 34 0
South Africa +27 11 570 1840
Spain +34 914 845 965
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC

Part of Thermo Fisher Scientific

ASMS13_W617_RViner _E 06/13S