Sensitive and Accurate Quantitation of Phosphopeptides Using TMT Isobaric Labeling Technique

Xiaoyue Jiang[1], Ryan Bomgarden[2], Joseph Brown[1], Rosa Viner[1], Andreas R Huhmer[1]
[1]Thermo Fisher Scientific, San Jose, CA; [2]Thermo Fisher Scientific, Rockford, IL

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ABSTRACT

Standard mass spectrometry (MS) instrument methods used for phosphopeptide analysis in multiplexed quantitative workflows suffer from lower peptide identification rates and quantitative accuracy compared to unenriched, non-phosphorylated peptides. Using two standard multiplexed phosphoproteome samples, we developed and optimized new acquisition methods which result in more phosphopeptide identifications with less ratio distortion compared to traditional methods. We also applied these improved methods to a large-scale study of phosphorylation levels in A549 cell lines after insulin and IGF-1 treatment.

INTRODUCTION

New advances in MS enable comprehensive characterization and accurate quantitation of complete proteomes. The most accurate isobaric mass tagging (e.g., Tandem Mass Tag™ (TMT™)) quantitation on high dynamic range complex mixtures can be accomplished by employing the Synchronous Precursor Selection (SPS) MS^3 method available on the Thermo Scientific™ Tribrid™ MS family of instruments. However, when applying this method to phosphopeptide quantitation, the number of phosphopeptide identifications drops significantly, due to differences in phosphopeptide fragmentation during MS/MS. In this study, we developed and optimized several instrument methods to address this limitation with the goal of providing high phosphopeptide identifications as well as accurate quantitation.

MATERIALS AND METHODS

Sample Preparation

For one sample, HeLa cells were lysed, digested and labeled with Thermo Scientific™ TMT10plex™ reagents before mixing at ratios of 16:8:4:2:1:1:2:4:8:16. Yeast digest (Promega) was labeled with the last 5 channels mixed equimolar (0:0:0:0:0:1:1:1:1:1) and spiked into the aforementioned TMT-labeled HeLa digest sample. For the second sample, A549 cells were serum starved overnight before stimulation with 100nM insulin or 100ng/ml IGF-1 for 15 min. Following treatment, cells were lysed, digested and labeled with TMT10plex reagents in triplicate or pooled for a reference channel (control: 126, 127N, 127C; insulin-treated: 128N, 128C, 129N; IGF-1 treated :129C, 130N, 130C; pooled sample:131). Both samples were further enriched for phosphopeptides using a Pierce™ Fe-NTA resin. Peptide concentrations were measured using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay before LC-MS analysis.

Table 1. Different instrument method settings. OT: orbitrap; IT: ion trap; MSA: multistage activation; NL: neutral loss

	MS^2	SPS MS^3	MSA SPS MS^3	NL trigger MS^3
dd MS^2	HCD OT	CID IT	CID OT	CID IT
Isolation width (Th)	0.7	0.7	0.7	0.7
Collision Energy	38	35	35	35
MSA	-	-	Yes/NL mass 97.9763	-
Resolution	60K	Turbo	30K	Turbo
Ion Injection Time (ms)	105	50	60	50
MS^3 filters				
Precursor selection Range	-	400-1200	400-1200	-
Precursor Ion Exclusion	-	-18 to +5	-18 to +5	-
Isobaric Tag Loss Exclusion	-	TMT	TMT	-
Targeted Loss	-	-	-	97.9763/79.9658
dd MS^3		HCD OT	HCD OT	HCD OT
Isolation Width	-	2	2	2
# of Notches	-	10	10	1
Collision Energy	-	65	65	38
Resolution	-	60K	60K	60K
Scan Range	-	100-500	100-500	100-2000

Liquid Chromatography and Mass Spectrometry

A Thermo Scientific™ EASY-nLC™ 1000 UPLC system and Thermo Scientific™ EASY-Spray™ source with a 50cm EASY-Spray Column was used to separate peptides with acetonitrile 7% to 25% over 190 min, 25% to 60% over 25 min, at a flow rate of 300 nl/min. An injection of 1ug of phosphopeptides was analyzed on a Thermo Scientific™ Orbitrap™ Fusion™ Tribrid™ MS or Orbitrap Fusion Lumos™ MS. The detailed instrument methods are shown in Table 1.

Data Analysis

The LC-MS data were analyzed using Thermo Scientific™ Proteome Discoverer™ software v.2.1 with the SEQUEST® HT search engine using carbamidomethylation(C) and TMT6(K, N term) as static modifications. Dynamic modifications included oxidation(M) and phospho (STY). Data were searched against a UniProt human and yeast database with a 1% FDR criteria using Percolator. Two technical replicates were analyzed for each sample with the average and standard deviation reported. All the numbers were filtered for human. ptmRS was used to localize phosphorylation sites. A probability of 90% or higher was considered as a confident phosphorylation site.

RESULTS

The large scale protein quantitation using TMT technology has been widely adopted with the MS^3 scan function and the SPS available on Orbitrap Fusion and Fusion Lumos MS, enabling more accurate quantitation by eliminating the co-isolation of contaminating ions [1] (Figure 1a and 1b). However, this improvement in quantitative accuracy using the SPS MS^3 method typically results in ~25% loss of unique peptide identifications compared to MS2 methods, due to longer instrument cycle times.

The quantitative analysis of phosphoproteomes is of keen interest to biologists because of the significance of phosphorylation in numerous cellular processes. In addition to quantifying the extent of protein phosphorylation, the correct localization of phosphorylation modification sites is equally important to understand protein function. While the SPS MS^3 method typically identifies ~75% of peptides compared to the less accurate HCD MS^2 method for unenriched peptides, only 66% is observed for phosphopeptides when comparing the same methods (Figure 2). Even worse, the phosphopeptides with confident localization identified from SPS MS^3 method were roughly half of those identified from MS^2 method.

We hypothesized that the loss in phosphopeptide identifications could be attributed to the specific characteristics of phosphopeptide fragmentation of MS^2 methods compared to SPS MS^3 methods. While MS^2 method uses HCD for peptide fragmentation and identification, the SPS MS^3 method uses CID fragmentation. For our highly enriched (>98%) phosphopeptide samples, over 66% of CID spectra contained a neutral loss (NL) peak as the base peak. In contrast, only 18% of NL peaks were observed as base peaks for HCD spectra (Figure 1c vs 1a). The strong presence of the neutral loss peak in CID results in the loss of sequence information, potentially compromising the ability to identify the phosphopeptide sequence or localize phosphorylation to a specific residue [2].

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Figure 1. Schematic of different instrument methods. Blue dots are fragment ions selected for MS3. Red peaks represent interference.

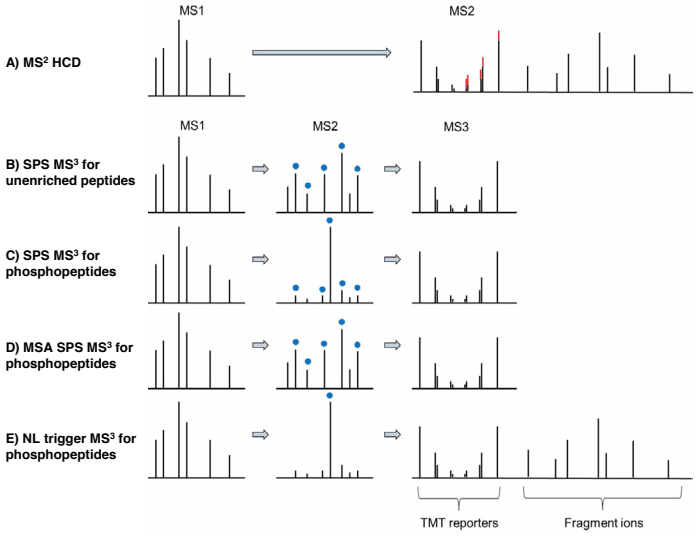
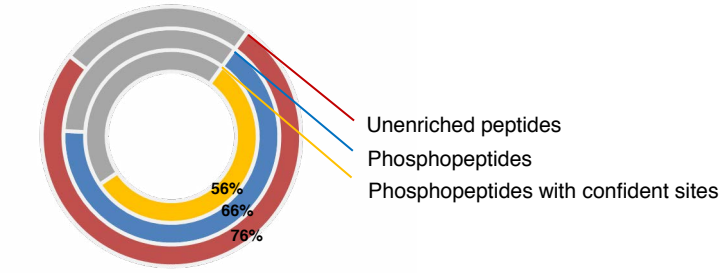


Figure 2. Percentage of identified peptides in SPS MS³ method compared to those identified from HCD MS² method



Multistage activation SPS MS³

Previously, an extra multistage activation (MSA) step to improve peptide fragmentation and phosphoyrlation site localization for ion trap based instruments has been developed [3]. MSA involves simultaneous activation of the precursor ion and the resultant neutral loss product ion during a single CID-MS/MS event. Therefore, the pseudo-MS³ product ion spectrum contains a "composite" of the product ions generated by fragmentation of both the precursor ion and the initial neutral loss product ion (Figure 1d). Notably, as the spectrum gets more complex, we found that utilizing the Orbitrap analyzer for detecting MSA CID MS² spectra was better than the ion trap analyzer as seen by an increase in phosphopeptide identifications and site localization (Figure 3). By combining MSA with the SPS MS³ method and using the Orbitrap analyzer as a detector for phosphoproteomics quantitation, we observed a16% increase in phosphopeptide identifications and 30% more phosphopeptides with confidently localized sites compared to the standard SPS MS³ method (Figure 4). In addition to improved peptide identifications, this method also provided a very high peptide quantitation rate (96%), similar to what is typically observed with the HCD MS² method (-7%) (Figure 5), while maintaining high quantitative accuracy (Figure 6).

Figure 3. The comparison of Orbitrap and ion trap analyzers for identifying phosphopeptides and the phosphopeptides with confident sites.

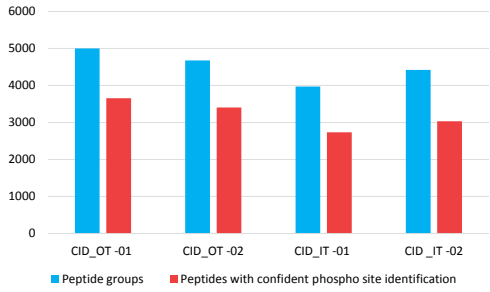


Figure 4. Improvement of MSA SPS MS³ and NL trigger methods over classic SPS MS³ method for phosphopeptide identification.

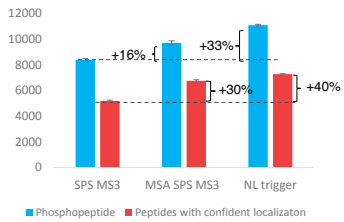
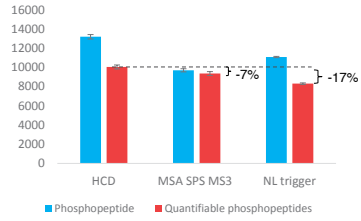


Figure 5. The number of phosphopeptides identified and quantified using HCD MS², MSA SPS MS³ and NL trigger methods.



Neutral loss triggered method

As stated previously, due to dominant neutral loss observed for some phosphopeptides spectra, the fragment ions are not sufficient for peptide identification or confident phophorylation site localization. Using a neutral loss triggered MS³ event with moderate collision energy (HCD CE38) allowed us to obtain both the sequence information and the quantitation from MS³ spectrum (Figure 1e). The quantitation is accurate since the MS³ reporter ions are generated from a unique NL MS² fragment, which already has co-eluting interference removed (Figure 6c). Our results from this method were consistent with a recent report showing that selecting single notch method for phosphopeptide provides the most accurate quantitation[4]. An additional benefit of the neutral loss trigger method is that both MS² spectra (CID) and MS³ spectra (HCD) are used for peptide identification, greatly improving the identification rate over the standard SPS MS³ method. Overall, the phosphopeptide identification and site localization improved 33% and 40%, respectively (Figure 4). As only a single notch is used for generating reporter ions for the quantitation, the sensitivity of this method is lower compared to MSA SPS method, but only 17% lower than HCD MS² method (Figure 5).

Figure 6. Distributions of TMT ratios corresponding to human phosphopeptide without interference (126/128N, blue trace), and human phosphopeptide with interference (131/129C, red trace). Expected Ratio is 8:1.

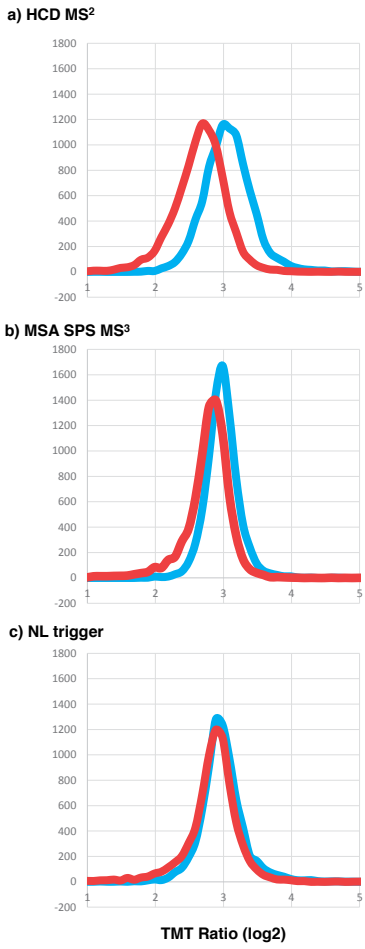
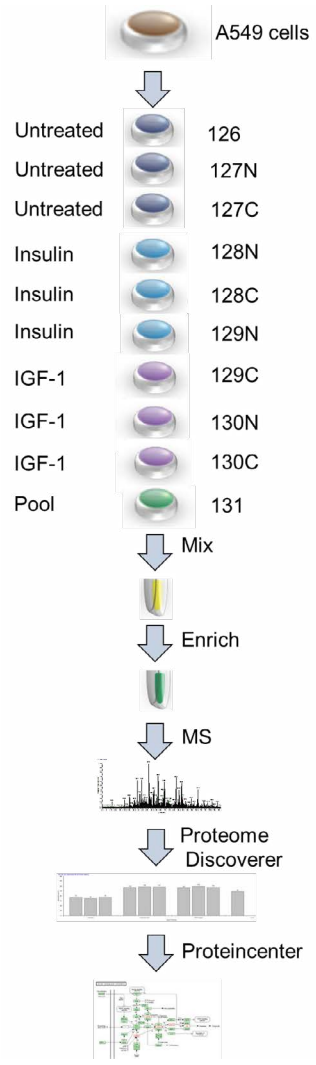


Figure 7. Workflow for phosphoproteome characterization



The two new developed methods were applied to a large scale phosphoproteome characterization in A549 cell line upon insulin and IGF-1 treatments as illustrated in Figure 7. Overall, 3,378 protein groups and 12,465 phosphopeptides were identified using both methods of which 10,436 were quantifiable. Reproducibility among replicates was high with over 94% of protein groups quantified with standard error of 20% or less in triplicates. Differences in phosphorylation among treatments were scaled for heatmap visualization (Figure 8). The accurate measurement enabled us to map regulated phosphorylation sites to numerous signaling pathways including the longevity regulating pathway, mTOR signaling pathway and AMPK signaling pathway. IGF-1 stimulates IGF1 receptor that insulin treatment cannot trigger (Figure 9).

Figure 8. Relative abundance of phosphopeptides for different conditions. A 1.25-fold change up or down is used as the threshold for differences in regulation.

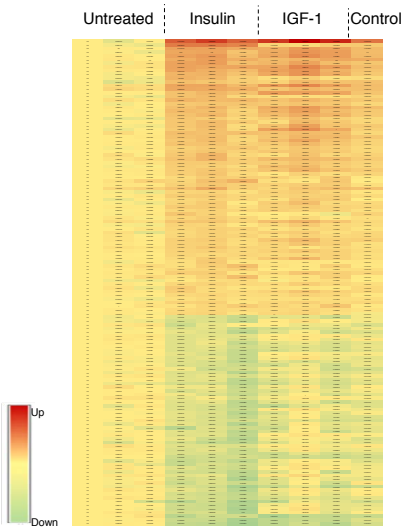
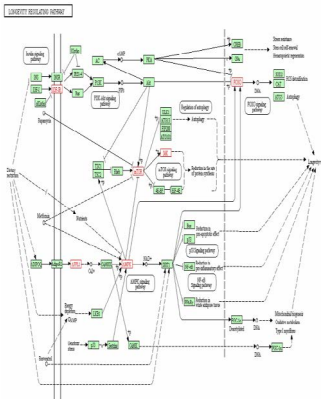


Figure 9. Longevity regulating pathway triggered by the stimulation of IGF-1.



CONCLUSIONS

- Standard SPS MS3 methods underperform for phosphopeptide analysis compared to unmodified peptides.
- Two novel MS3 methods using multistage activation or a neutral loss trigger were developed and optimized to achieve higher numbers of phosphopeptide identifications and site localizations.
- Application of these methods to large scale phosphoproteome analysis identified subtle changes in insulin and IFG-1 signaling with improved phosphoyrlation site localization confidence.

REFERENCES

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3. Schroeder M., Shabanowitz J., Schwartz J., Hunt D., Coon J. 2004. *Anal. Chem.,* **76:**3590–3598.
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