Quantitative Peptide Assay for Optimized and Reproducible Sample Preparations

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Overview

Purpose: To determine how a quantitative peptide assay could improve the typical bottom-up mass spectrometry applications to answer biological questions.

Methods: An absorbance-based colorimetric assay, based on the reduction of copper, was used to determine peptide concentration at each sample preparation step.

Results: Sensitive and accurate quantitation to monitor changes in protein expression in key signaling pathways is achieved.

Introduction

New advances in mass spectrometry enable comprehensive characterization and accurate quantitation of complete proteomes. Despite the rapid advances in analytical instrumentation and data processing, the quality of the generated data depends to a large extent on the upstream sample preparation techniques. The ability to deliver accurate quantities of peptide material for mass spectrometric analysis in a reproducible manner would lead to a significant reduction in variability of the results by standardizing determination of accurate peptide amounts as part of the sample preparation in the proteomic workflows. In this study we utilized a peptide quantitation assay to monitor the peptide concentration at each step of a typical bottom-up proteomics workflow, including labeling with Thermo Scientific™ Tandem Mass Tag™ (TMT) reagents and off-line fractionation steps.

Methods

Sample Preparation

A549 human cells were serum starved overnight and stimulated for 15 minutes with insulin or 15 minutes IGF-1. Cells were lysed and digested using the Pierce™ Mass Spec Sample Prep Kit for Cultured Cells. Protein and peptide concentrations were determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit and the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay. Concentrations were normalized before labeling samples with Thermo Scientific™ TMT6plex™ labeling reagents. Labeled samples were fractionated using the Pierce™ High pH Reversed-Phase Peptide Fractionation Kit. The peptide concentrations were measured at each of above experimental steps. Labeled samples were further enriched using Thermo Scientific™ Pierce™ Fe-NTA Phosphopeptide Enrichment Kit for pathway analysis (Figure 1).

Liquid Chromatography and Mass Spectrometry

A Thermo Scientific™ EASY-nLC™ 1000 UPLC system and Thermo Scientific™ EASY-Spray™ source with 50 cm EASY-Spray Column was used to separate peptides with 30% acetonitrile gradient over 120 min, at a flow rate of 300 nl/min. The A549 cell lysate of different conditions (100ng) and TMT labeled lysate fractions (500ng) were analyzed on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. Phosphopeptide enriched TMT labeled sample (1ug) was analyzed on a Thermo Scientific™ Q Exactive™ Plus mass spectrometer. LC and MS settings are shown in Table 1.

Data Analysis

The LC-MS data for protein identification were analyzed using the pre-released Thermo Scientific™ Proteome Discoverer™ software v.2.1. with the SEQUEST HT search engine. Data were searched against a Uniprot human database with a 1% FDR criteria using Percolator software.

FIGURE 1. Experimental workflow.

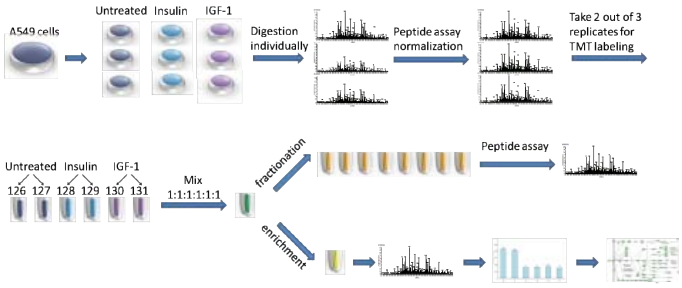

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TABLE 1. LC, MS and database search parameter settings for different samples and instruments.

	Direct Digest on Orbitrap Fusion	TMT6 Labeled Fractions on Orbitrap Fusion	TMT Labeled Phosphopeptides on Q Exactive Plus
LC gradient	5-25%B in 90min 25-35%B in 10min	10-25% in 85min 25-60% in 20min	3-22% in 165min 22-35% in 20min
Full MS	OT	OT	OT
Resolution	120K	120K	70K
Target value	4e5	4e5	3e6
Max injection time	50	50	50
Top N	3 sec	3 sec	15
MS2	Ion trap HCD	Ion trap CID	OT HCD
Isolation mode	Quadrupole	Quadrupole	Quadrupole
Isolation width	1.6	1.2	0.7
NCE	28	35	32
Resolution	Rapid	Turbo	35K
Target value	1e4	1e4	1e5
Max injection time	70ms	70ms	120ms
MS3 SPS		OT HCD	
Isolation width		2	
NCE		65	
Resolution		60K	
Target value		1e5	
Max injection time		120ms	
Search parameters	SequestHT	SequestHT	SequestHT
Precursor tolerance	10ppm	10ppm	10ppm
Fragment tolerance	0.6Da	0.6Da	0.02Da
Static	Carbamidomethyl(C)	Carbamidomethyl(C) TMT6 (K, N term)	Carbamidomethyl(C) TMT6 (K, N term)
Dynamic	Oxidation (M)	Oxidation (M) Phospho (S,T,Y)	Oxidation (M) Phospho (S,T,Y)

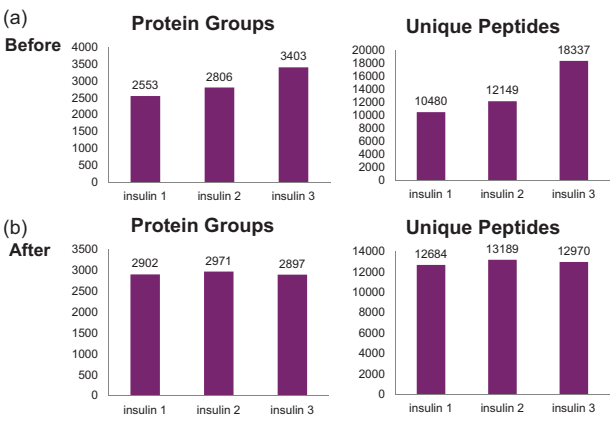
Results

Peptide Quantitation Before LC-MS Improves Data Reproducibility

Variations in peptide and protein identifications can come from both sample preparation and LC-MS instrumentation. Advances in instrumentation have improved both LC reproducibly and MS speed/sensitivity, which has greatly reduced the variation from the instrument platform. Variation in biological replicates can come from the samples themselves but is largely dependent on reproducible sample preparation.

In a typical bottom up experiment, while the protein concentration is routinely measured using BCA Protein assays before the digestion, final digested peptide concentrations loaded on column may still be highly variable due to difference in sample preparation and clean up. To assess this, we analyzed replicate samples (100ng) using column loads estimated from the original BCA protein assay concentration (Figure 2a). We observed up to 30% CV in peptide identification and 15% CV in protein identifications among replicates (Figure 2a). Using a peptide assay to measure the concentration of the final digested samples revealed up to a 75% difference in concentrations in the triplets. After normalization based on the peptide assay concentration, less than 2% variability in peptide and protein identifications were observed (Figure 2b), These data demonstrate the need for accurate determination of peptide concentration before LC-MS analysis.

FIGURE 2. The number of unique protein groups and peptides identified when injecting 100ng of insulin-treated triplicates on an Orbitrap Fusion MS before and after normalization.



Peptide Assay Optimizes the Sample Loading on Mass Spectrometer

With the ability to accurately monitor the peptide concentration, the sample loss in each preparation step can be easily monitored. We compared the BCA protein assay and colorimetric peptide assay and determined that sample loss during digestion step was less than 10%. We also observed the fractionation procedure can recover ~70% of the loaded peptides (Figure 3) suggesting that sample clean up is the main source of sample loss.

In addition, peptide concentration measurement informs us on the sample loading for each fraction. Previously, the injected amount was based on the theoretical assumption, without considering the sample loss in each experimental step and the difference in peptide concentration among fractions. Here, we utilized the colorimetric peptide assay to measure the peptide concentration for each fraction (Figure 3). While the theoretical concentration for each fraction is 350ng/µl, the actual loading concentration varies from 117ng/µl to 561ng/µl. One interesting observation using the colorimetric assay was that the measurement for the first fraction was much higher than expected suggesting potential interference from excess, un-reacted TMT reagent. To eliminate interferences, we included additional clean up step using 5% of AcN.

After adjusting the injection volume based on peptide concentration for each fraction, we observed increased peptide identification for 7 out of 8 fractions, with the last two fractions showing largest increase (Figure 4). This resulted in a total of 38,133 peptide groups and 5,059 protein groups identified with Orbitrap Fusion MS SPS method.

FIGURE 3. The peptide concentration distribution across 8 fractions. The dotted line is the theoretical peptide concentration, if assuming peptide is equally eluted into each fraction. The overall sample recovery is ~70%.

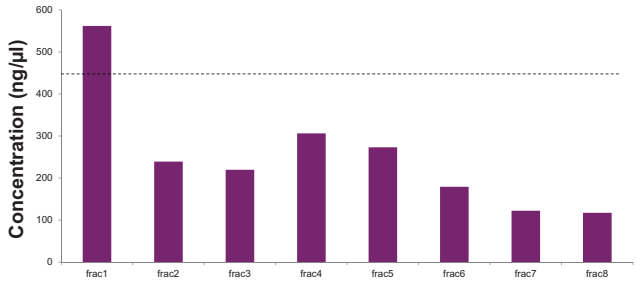
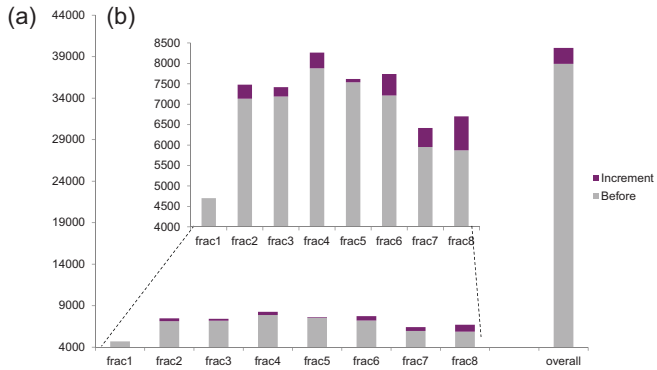


FIGURE 4. (a) Numbers of peptides identified when injecting 500ug fractions and overall combined results. (b) Zoom in of (a) for fractions. Original identification numbers are shown in grey. After normalization, the identification increments are highlighted in purple.



TMT6plex Quantitation and Pathway Analysis of Enriched Sample

Even though TMT6 labeling enables quantitation of > 99% of identified peptides and proteins, less than 1% of peptides are identified as phosphopeptides. Therefore, we used a FE-NTA phosphopeptide enrichment kit to enrich TMT6plex-labeled samples. We identified over 2,570 protein groups and 2,277 of them are phosphoproteins, with a 88% enrichment rate using the Q Exactive Plus MS. As expected, after 15 min stimulation with insulin or IGF-1 many phosphoproteins were shown to be regulated. This enabled the partial mapping of numerous important KEGG signaling pathways (Table 2). Overall, we found that the treatments quickly stimulates multiple cellular functions including DNA replication, RNA splicing, protein synthesis and cell division. In addition, we confirmed that insulin and mTOR signaling pathways are triggered as well.

TABLE 2. Functional annotated pathways found in DAVID for up-regulated and down-regulated proteins. A 1.25-fold change up or down is used as the threshold for differences in regulation.

	Pathway Mapped	Protein Count	P-Value
1	Ribosome	7	7.3E-3
2	Spliceosome	8	1.2E-2
3	Cell cycle	7	3.7E-2
4	Insulin signaling pathway	7	5.1E-2
5	Wnt signaling pathway	7	7.9E-2
6	Purine metabolism	7	8.3E-2
7	mTOR signaling pathway	4	8.4E-2

For example, proline-rich AKT 1 substrate 1, was found to be up-regulated for both treatments (Figure 5a). In contrast, reticulon-4 was more responsive to IGF-1, compared to insulin treatment (Figure 5b). Dihydropyrimidinase-related protein 2 was down-regulated for cells treated with insulin and IGF-1 (Figure 6a). In this protein, peptide TVTPASSAKTSPAK[512-525] with S517, S518 and S522 triple phosphorylated dropped significantly in intensity upon treatment, while the same peptide sequence TVTPASSAKTSPAK with S517 and S522 double phosphorylated did not show a change in abundance (Figure 6b and 6c), indicating the site S518 may play an important role in regulating the pathway. Although this S518 site has been identified in a few publications before [1,2], our data is the first to show this particular site is down regulated after both insulin and IGF-1 treatment.

FIGURE 5. Differential expression of key proteins in A549 cell lines determined by TMT reporter ion based quantitation. Results are presented as normalized weighted average protein abundances for two replicate's runs.

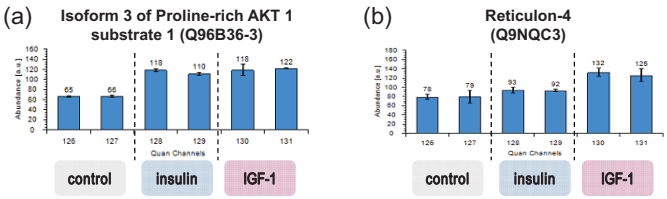
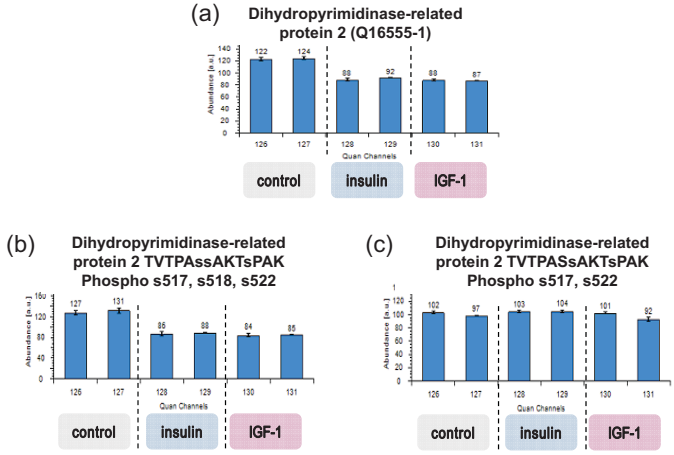


FIGURE 6. Differential expression of phosphosites in protein Dihydropyrimidinase-related protein 2 (Accession Q16555-1) upon stimulation. (a) Overall proteins was down regulated; (b) Abundance of triple phosphorylated peptide[512- 525] got down regulated; (c) Abundance of double phosphorylated form of the same peptide was unaffected.



Conclusions

- Using a peptide assay to monitor sample preparation enabled determination of sample loss for each sample preparation step
- Peptide concentration measurement before mass spectrometry analysis can improve biological replicate reproducibility, number of identifications and quantitative accuracy
- High pH reverse phase fractionation and phospho-peptide enriched results in a ~200% increase in number of proteins/peptides identified and quantified compared to direct analysis
- TMT relative quantitation and pathway analysis reveals differences in the regulation of insulin pathway signaling proteins after insulin and IGF-1 treatment

References

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