Large Scale EasyPep™ MS Sample Preparation for Phosphopeptide Enrichment Workflows

Ryan D. Bomgarden, Amarjeet Flora, Sergei Snovida, Penny Jensen, John C. Rogers **Thermo Fisher Scientific, Rockford, IL, USA, 61101**

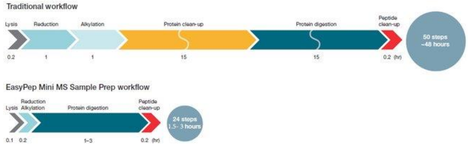
ABSTRACT

Phosphorylation is a critical post translational modification that modulates the function of numerous proteins, and recent advances in mass spectrometry (MS) instrumentation have enabled studying phosphorylation at proteome- wide scale in complex biological samples. However, due to the low abundance of phosphorylated peptides in protein digests, affinity-based phosphopeptide enrichment from milligrams of protein digest is required for MS detection and quantification. Recently, we developed a new, simplified sample prep kit containing pre-formulated reagents and a standardized protocol for processing 10 to 100µg protein samples in less than 2 hours. In this study, we assessed the scalability of our chemistry on larger protein amounts (>1mg) for subsequent phosphopeptide enrichment using immobilized metal affinity chromatography (IMAC).

INTRODUCTION

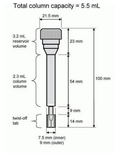
Sample Preparation is a crucial step in the proteomics workflow greatly impacts peptide and protein identification rates. Conventional workflows typically involve home brew buffers and protocols which rely on sonication for cell lysis in denaturing buffers (e.g. urea, GuHCl or SDS) for protein extraction followed by protein clean up (e.g. dialysis or precipitation) before overnight digestion using trypsin and peptide desalting (Figure 1). In addition to long processing times, these methods can be highly variable among labs leading to poor sample reproducibility. Recently, we developed an optimized and standardized workflow developed for simple, shot, convenient and easy-to-use MS sample preparation for proteins, cells, tissues, serum and plasma. Here, we assessed the scalability of our chemistry on larger protein amounts for phosphopeptide enrichment.

Figure 1: Comparison between conventional workflow and improved EasyPep standardized workflow



Due to the low stoichiometry of phosphorylated peptides in complex protein digests, phosphopeptide enrichment from large scale protein digests (>1mg) is typically required to identify and localize significant numbers of phosphopeptide sites by mass spectrometry. Although our optimized chemistry developed for significantly reduces both hands-on and total sample processing time, peptide clean-up using microcentrifuge spin columns is limited by the amount of resin and device volume. We assessed various centrifugation-based and vacuum-based column formats for peptide clean-up and identified a large scale format (as shown in Figure 2) containing up to 100mg of resin showed nearly identical performance in terms of peptide yield, phosphopeptide specificity, identification rates, alkylation efficiency and digestion efficiency compared to our EasyPep smaller spin column protocol (Figure 3).

Figure 2 : Dimensions of EasyPep large scale column



MATERIALS AND METHODS

Cell treatment

HeLa S3 cells were grown in sMEM media supplemented with 10% FBS, 1X Glutamax and 1% Pen/Strep. For phosphopeptide enrichment benchmarking, cells were treated with 0.1µg/ml nocodazole for 22 hours. For TMT-labeled samples, HeLa S3 cells were grown in serum starved media containing 0.1% charcoal stripped FBS for 18-20 hours before treatment with different conditions (no stimulation, stimulation with hEGF or IGF at 0.1µg/mL and 10µM Erlotinib) for 15 minutes as shown in Figure 7.

Sample Preparation

Cellular protein extracts were diluted in lysis buffer with a phosphatase inhibitor mixture. A universal nuclease was added to cellular extracts to reduce sample viscosity. Protein samples were heated at 95˚C for 10 minutes in the presence of combined reduction/alkylation solution before digestion using a trypsin/Lys-C protease mixture at 1:25, w:w. A mixed mode peptide clean-up procedure was used to remove detergent removal before IMAC enrichment using Thermo Scientific Pierce Hi-Select™ Fe-NTA phosphopeptide enrichment kit. Protein concentration was measured using Pierce™ Rapid Gold BCA Assay kit. Peptide concentration was determined using Pierce™ Quantitative Colorimetric Peptide Assay kit prior to LC-MS Analysis. The samples were labeled with Thermo Scientific™ TMT™ 11plex™ reagents or Thermo Scientific™ TMTpro 16plex reagents according to the manufactures protocol before sample clean up, phosphopeptide enrichment and high pH reversed phase fractionation.

LC-MS Analysis

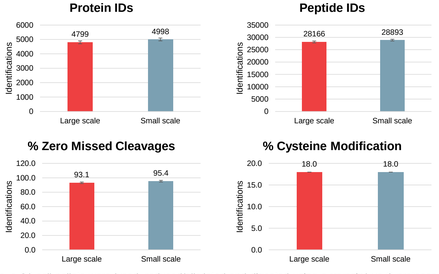
Triplicate protein digest samples (1µg per injection) were separated using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system using a 50 cm C18 Thermo Scientific EASY-Spray™ column with an acetonitrile gradient from 3% to 28% over 85 min, 28% to 45% over 30 min, at a flow rate of 300nL/min on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. A Thermo Scientific™ Orbitrap Fusion™ mass spectrometer was used with the same LC-MS conditions to analyze TMT-labeled samples.

Data Analysis

LC-MS data were analyzed using the SEQUEST® HT search engine in Thermo Scientific™ Proteome Discoverer™ 2.3 software using static carbamidomethyl (C), dynamic oxidation (M), TMT6plex or TMTpro (K, N-term), Phospho (S, T, Y) and deamidation (N, Q) modifications. Data were searched against the Uniprot human protein database and results were filtered using a 1% protein FDR threshold.

RESULTS

Figure 3 : Assessing performance of EasyPep large column compared to smaller spin column format

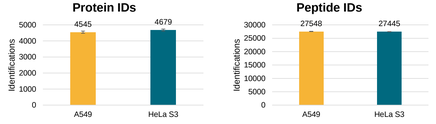


HeLa S3 cell pellets were lysed, reduced/alkylated and digested using a Trypsin/Lys-C protease mix followed by the mixed mode clean-up procedure. Protein digest (1µg) was analyzed by LC-MS and analyzed as described in the methods. The results demonstrated that the large scale column format showed nearly identical performance to the smaller spin column protocol.

Figure 4: Sample preparation of human plasma

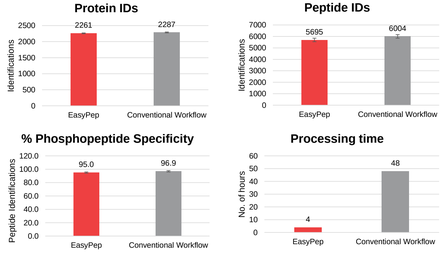
Human Plasma digest was prepared using EasyPep small scale and large scale column format. Human Plasma digest (1µg) was analyzed by LC-MS as described in the methods. The results demonstrated that the number of proteins and peptides identified with the EasyPep large scale column format were nearly identical to the EasyPep small scale column format.

Figure 5: Compatibility with different cell lines



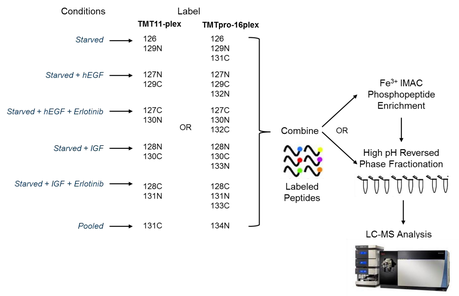
HeLa S3 and A549 cell pellets were lysed, reduced/alkylated and digested using a Trypsin/Lys-C protease mix followed by the mixed mode clean-up procedure. Protein digest (1µg) was analyzed by LC-MS and analyzed as described in the methods. The results demonstrated that our workflow is compatible with the various cell types yielding high protein/peptide identification rates.

Figure 6: Efficient sample preparation in less time



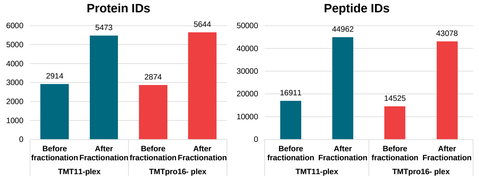
Nocodazole- arrested HeLa S3 cell pellets were processed using EasyPep and conventional workflow before IMAC enrichment. The number of protein/peptide identifications with EasyPep workflow was not significantly different than conventional workflow. These results demonstrate that an efficient sample preparation can be performed in 3-5 hours using our EasyPep workflow with 95% phosphopeptide specificity.

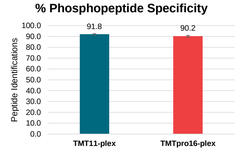
Figure 7: Schematic of the workflow



HeLa S3 cells were grown in a serum starved media and exposed to different conditions for 15 minutes as shown in the workflow. HeLa S3 cell pellets were lysed, reduced/alkylated, digested, labeled with Thermo Scientific™ TMT™ 11plex or TMTpro 16plex reagents, combined together before mixed mode cleaned-up for removal of detergent and excess, unreacted tag. Samples were enriched for phosphopeptides using Fe^{3+} IMAC columns followed by fractionation using the Pierce™ High pH Reversed-Phase Peptide Fractionation kit.

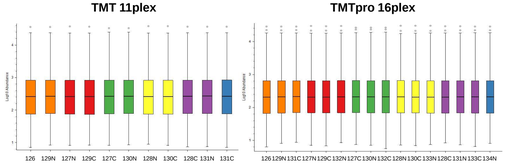
Figure 8 : Protein/Peptide Identifications from unenriched TMT11plex or TMTpro 16plex labeled samples before and after high pH reversed-phase fractionation





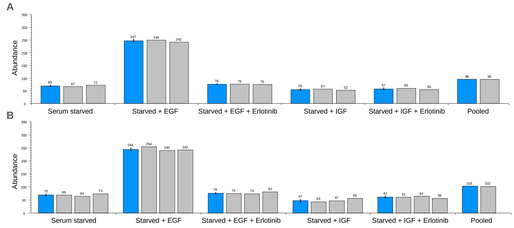
HeLa S3 cell pellets were processed using EasyPep kit and labeled with TMT 11plex or TMTpro 16plex reagents as shown in Figure 7 followed by the mixed mode clean-up procedure, phosphopeptide enrichment and high pH reversed phase fractionation. Protein and peptide identifications are similar using TMTpro reagents compared to TMT reagents. No significant differences were observed for phosphopeptide specificity between the two tagged samples demonstrating compatibility of the new tag with phosphopeptide enrichment workflows.

Figure 9: Multiplex quantitation of different sample conditions



Overall, the multiplex quantitation of samples was the same of across all conditions using TMT 11plex or TMTpro 16plex reagents (Figure 9). Although both multiplex tags showed similar number of unenriched protein and peptide identified by LC-MS (Figure 7), fewer phosphopeptides were quantified with TMTpro-labeled samples which requires further method optimization (data not shown). Both multiplex sets were used to identify and quantify an EGFR peptide which was phosphorylated upon EGF treatment and inhibited by Erlotinib (Figure 10).

Figure 10: Example of EGFR phosphopeptide GSTAENEpYLR quantified using TMT 11plex (A) and TMT 16plex (B) reagents after phosphopeptide enrichment and high pH reverse phase fractionation.



CONCLUSIONS

- We identified a column for a large scale column format that showed nearly identical performance in terms of peptide yield, phosphopeptide specificity, reduction/alkylation efficiency, digestion efficiency and identification rates compared to smaller spin column protocol.
- Our workflow is compatible with several sample types including different cell lines, plasma and other tissues with high reproducibility.
- Our EasyPep chemistry is readily adaptable to large-scale enrichment for phosphoproteome mass spectrometry analysis.
- Our sample preparation is compatible with isobaric labeling reagents such as TMT and TMTpro reagents for relative protein quantitation.

TRADEMARKS/LICENSING

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