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Adapting EasyPep™ MS Sample Preparation for 96-well Automated Liquid Handling Systems

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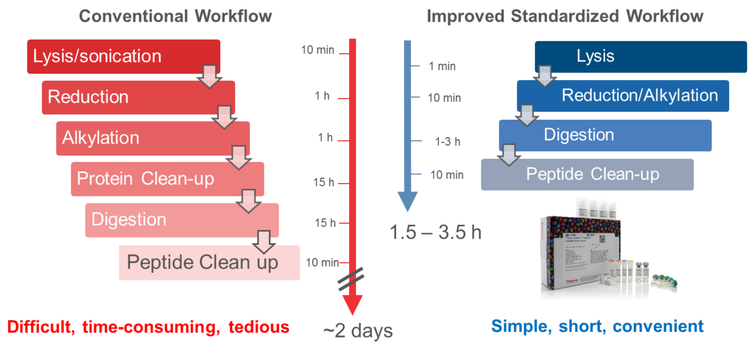
ABSTRACT

Advances in mass spectrometry (MS) instrumentation has enabled routine analysis of complex protein samples. However, sample preparation methods are not standardized with many protocols taking 8-24 hours in addition to suffering from low peptide yields, poor digestion efficiency and low reproducibility. Here, we describe a simplified sample prep kit containing pre-formulated reagents and a standardized protocol that can be used to efficiently process 10µg to 100µg protein samples in less than 2 hours. In this study, we evaluated the scalability, compatibility, and reproducibility of this sample preparation kit compared to previous published methods. Our new standardized workflow yielded 10-20% higher number of peptides and proteins with lower missed cleavages (<90%) compared to other commercial MS sample prep kits and protein digest standards. We also developed a new 96-well filter plate format to support higher sample processing throughput amenable to the use with automated liquid handling system for sample preparation, TMT labeling, and peptide clean-up. This format showed nearly identical performance in terms of peptide yield, identification rates, alkylation efficiency and digestion efficiency compared to the manual spin column protocol with better reproducibility among replicates.

INTRODUCTION

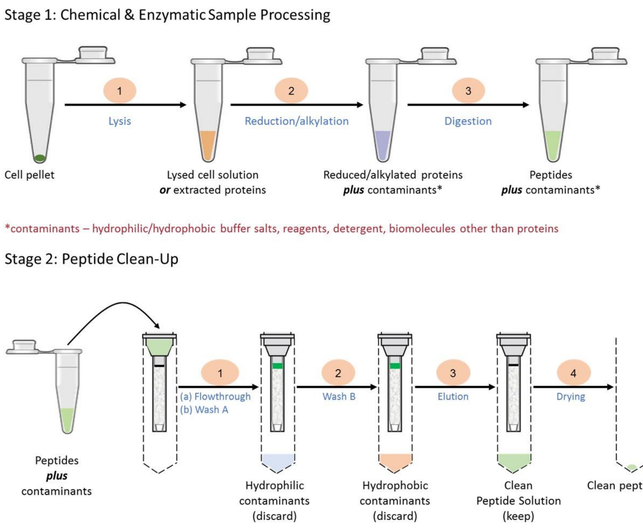
Sample preparation is a crucial step in the proteomics workflow greatly impacts peptide and protein identification rates. Conventional workflows typically involve home brew buffers and protocols which rely on sonication for cell lysis in denaturing buffers (e.g. urea, GuHCl or SDS) for protein extraction 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. Here, we introduce an optimized and standardized workflow developed for simple, short, convenient and easy-to-use MS sample preparation for proteins, cells, tissues, serum and plasma.

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



Our optimized protocol enables efficient and reproducible processing of cultured mammalian cells and tissues, reduces hands on time to less than 30 minutes with total sample processing time from intact cells to cleaned-up peptides under 1.5 hours. This kit includes universal nuclease which eliminates the need of sonication for cell and tissue lysis. Addition of universal nuclease is not required for purified protein and plasma samples. The samples are then denatured and reduced using a reduction/alkylation solution at 95˚C for 10 minutes followed by a combined trypsin/Lys-C digestion at 37˚C for 1-3 hours. The digestion is then acidified with Stop Solution before peptide clean-up using a mixed-mode resin in a spin column format for detergent and contaminant removal.

Figure 2 . Schematic of the EasyPep MS Sample Prep Kit Workflow.



The first stage in our standardized workflow is the chemical and enzymatic processing of the sample. It includes the lysis, denaturation, reduction and alkylation of the protein sample. The reduced and alkylated sample is then digested using a Trypsin/Lys-C protease mix for 1 to 3 hours. The second stage is the peptide clean-up. The protein digest sample is cleaned-up using the pre-formulated buffers and a mixed-mode resin to get rid of the hydrophilic and hydrophobic contaminants. The detergent-free peptides are eluted using an elution buffer followed by MS analysis.

MATERIALS AND METHODS

Sample Preparation

HeLa S3 cells were grown in sMEM supplemented with 10% FBS, 1X Glutamax and 1% Pen/Strep. HEK293 and A549 cells were grown in MEM supplemented with 10% FBS and 1% Pen/Strep. Human Plasma and Serum were obtained from BioReclamation, LLC. Mouse tissues were obtained from Pelfreeze.

Protein, cell, tissues, plasma and serum samples were processed using the Pierce™ Mass Spec Sample Kit for Cultured cells (i.e. conventional workflow) or the Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit. For liquid handling experiments, a 96-well filter plate format processed using a vacuum manifold was used for peptide clean up. Protein concentration was measured using Pierce™ Rapid Gold BCA Assay kit. Peptide concentration was determined using a Pierce™ Quantitative Colorimetric Peptide Assay Kit prior to LC-MS Analysis

LC-MS Analysis

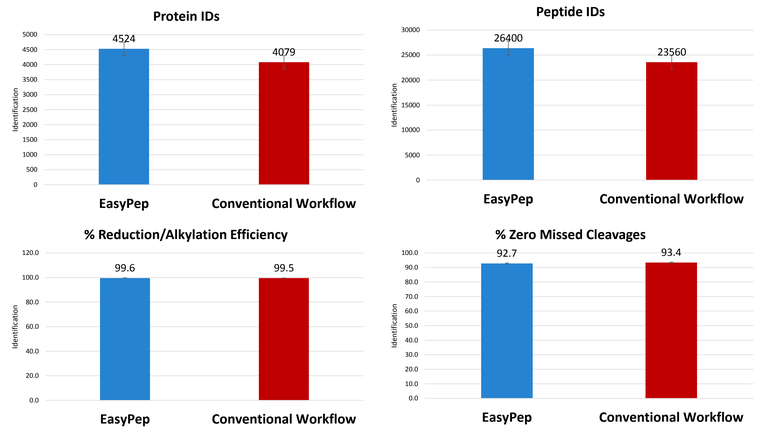
Triplicate protein digest samples (1µg per injection) were separated using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system using a 50cm C18 Thermo Scientific™ EASY-Spray™ column with an acetonitrile gradient from 5% to 25% over 80 min, 25% to 50% over 40 min, at a flow rate of 300 nL/min. A Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer was used for acquiring the top 20 MS/MS spectra in DDA mode.

Data Analysis

LC-MS data were analyzed using the SEQUEST® HT search engine in Thermo Scientific™ Proteome Discoverer™ 2.2 software using static carbamidomethylation (C), dynamic oxidation (M) and deamidation (N, Q) modifications. Data were searched against the Uniprot human protein database and results were filtered using a 1% protein FDR threshold.

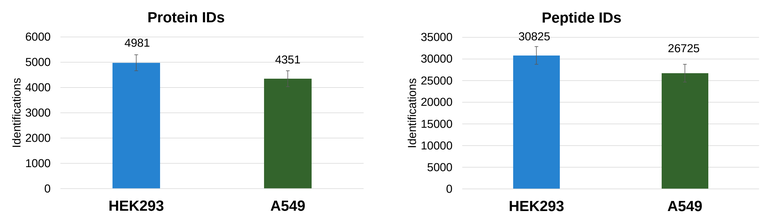
RESULTS

Figure 3. Efficient sample preparation in less time with higher identification rates



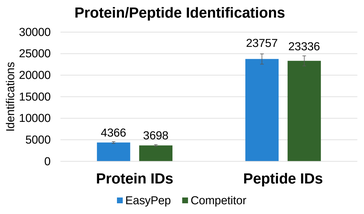
HeLa S3 cell pellets were lysed, reduced, alkylated and digested using a Trypsin/Lys-C protease mix followed by the detergent removal using the mixed mode peptide clean-up columns. Protein digest (1µg) was analyzed by LC-MS and processed as described in the methods. The results in this figure show that our standardized workflow enables efficient 3 hour sample preparation with a higher protein/peptide identification rates as compared to conventional workflow which used a 2.5 day protocol.

Figure 4. Compatibility with different cell types



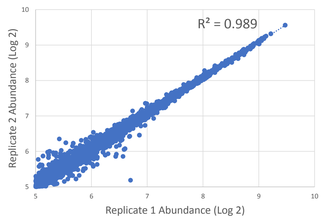
HEK293 and A549 cell pellets were prepared using our standardized and optimized workflow described in Figure 2. Protein digest (1µg) was analyzed by LC-MS and processed as described in the methods. The results demonstrate that our standardized workflow is compatible with the various cell types yielding high protein/peptide identification rates.

Figure 5. Assessing Workflow Scalability



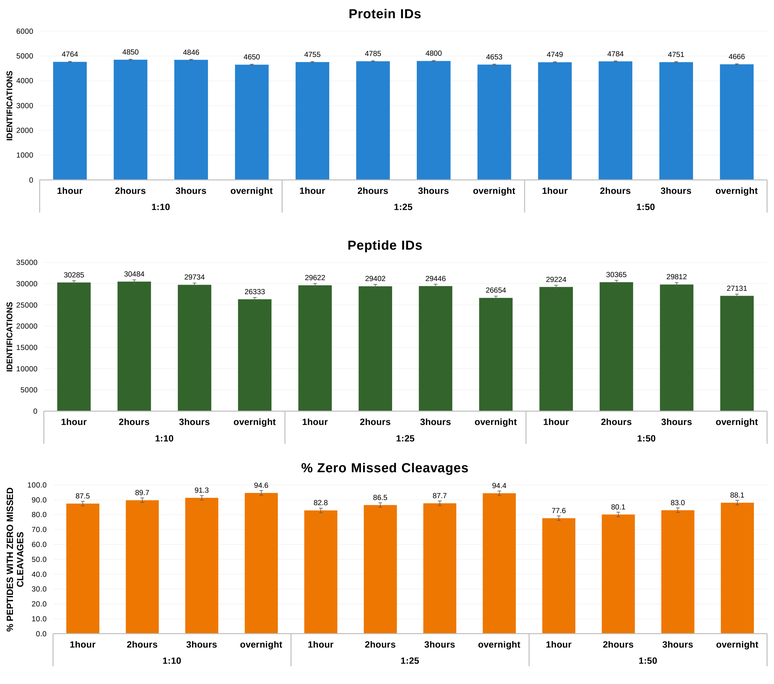
10ug of the protein sample was reduced, alkylated and digested followed by the clean-up on peptide clean-up columns. The results demonstrate that an efficient sample preparation can be performed using our EasyPep kit with higher protein/peptide identifications.

Figure 6. Assessing Workflow Reproducibility



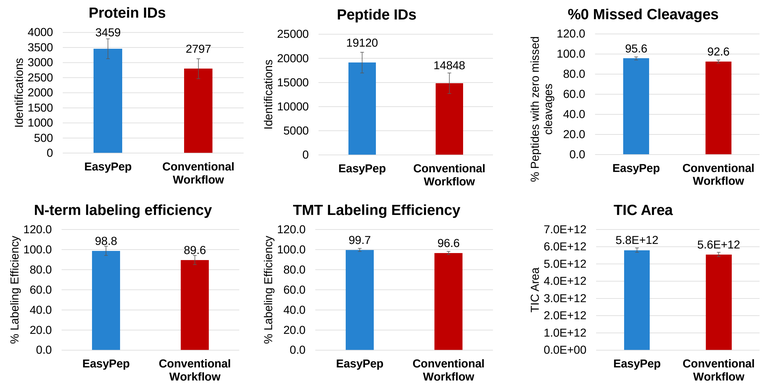
Replicate HeLa S3 cell pellets were processed indently using EasyPep workflow. Label free quantification of relative protein abundance showed excellent reproducibility with an R^2 of 0.989 and CVs < 5% between replicates.

Figure 7. Protein/Peptide identifications and enzymatic digestion efficiency for various enzymatic to protein ratios over time.



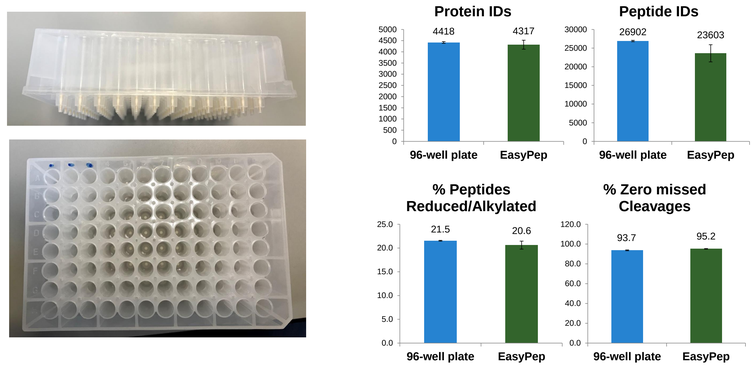
The reduced and alkylated protein sample from HeLa S3 cell pellets was subjected to enzymatic digestion over a time-course of 1 hour, 2 hours, 3 hours and overnight using an enzyme to protein ratio of 1:10, 1:25 and 1:50. The results described that an efficient digestion can be carried out in 1-3 hours. Overnight digestion resulted in higher digestion efficiency with less protein/peptide identifications as compared to the few hours digestion.

Figure 8: TMT labeling with EasyPep and Conventional workflow



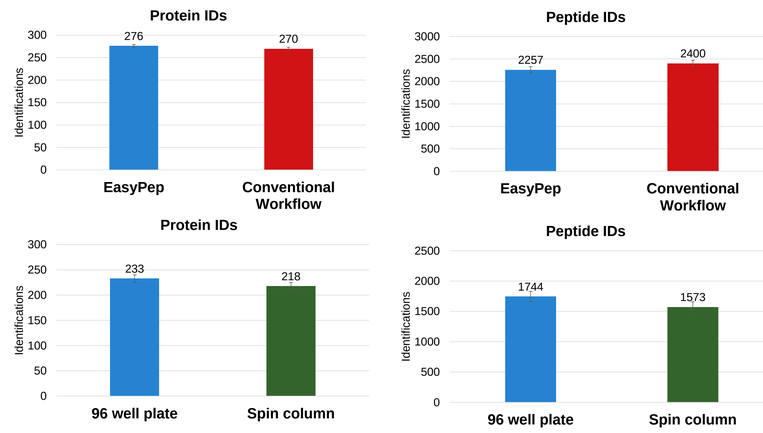
HeLa cell pellets were lysed, reduced, alkylated and enzymatically digested. The samples were labeled with TMT reagents after digestion and cleaned-up using mixed-mode peptide clean-up procedure. As shown in the figure, our EasyPep workflow is compatible with TMT labeling reagents and yielded 10-20% higher protein and peptide IDs with lower missed cleavages and labeling efficiency of >99%.

Figure 9. 96-well filter plate for EasyPep sample prep automation



HeLa S3 cell pellets were lysed, reduced, alkylated and digested using a Trypsin/Lys-C protease mix with 100µg of digest cleaned-up using EasyPep spin columns or a 96-well filter plate containing the same resin and buffers. Protein digest (1µg) was analyzed by LC-MS and processed as described in the methods. The results in this figure show that our standardized workflow enables amenable to 96-well formats with similar protein and peptide identification rates and excellent reproducibility between replicate samples.

Figure 10. Sample Preparation of Human Plasma using Different Methods



Human Plasma digest (0.5µl) was prepared using EasyPep and conventional workflow. Manual spin column and 96-well filter plate peptide clean up was also evaluated as described in the methods. The number of proteins and peptides identified for EasyPep workflow were nearly identical to the conventional workflow with significantly less processing time.

CONCLUSIONS

- Our new kit provides a superior method in terms of time saved, peptide/protein identification rates, and reproducibility compared to previous proteomic methods and greatly simplifies proteomic sample preparation for protein identification and quantitation.
- Our standardized workflow is compatible with several sample types including cell lines and mouse tissues, purified proteins, plasma and serum with high reproducibility (CVs <5%) and low missed cleavages (<90%).
- Our sample preparation chemistry is compatible with isobaric labeling reagents such as Tandem Mass Tags (TMT) for the relative protein quantification
- Peptide clean up using a 96-well filter plated shows equal or better peptide/protein identification rates and reproducibility (CVs <1%) compared to manual spin columns for HeLa cell pellets and plasma samples.

TRADEMARKS/LICENSING

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