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Optimization of Sample Preparation and Off-Line High pH Reversed-Phase Fractionation for TMTpro-labeled Proteomics Samples.

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ABSTRACT

Advances in mass spectrometry (MS) instrumentation have 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. We have recently introduced several easy-to-use sample preparation kits, based on the Thermo Scientific™ EasyPep™ sample preparation workflow that enables quick and reproducible proteomic sample preparation form cells, tissue, and fluids, while maintaining excellent sample quality metrics, such as peptide yields, digestion and chemical labeling efficiency, and high protein identification numbers. Together with the use of the new Thermo Scientific™ TMTpro™ tandem mass tag reagent, which allows multiplexing of up to sixteen samples in a single batch, researchers can quickly process and analyze hundreds of samples in a matter of days. Here, we describe a robust workflow for preparing complex proteomic samples which includes labeling with the tandem mass tag reagents, efficient clean-up, and off-line high pH reversed-phase for a comprehensive comparative analysis. 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.

INTRODUCTION

Effective removal of the excess chemical tags generally improves the LC-MS analysis of proteomic samples and reduces the overall maintenance times for the instruments due to accumulation of the non-volatile chemical matter in the source. The goals of this study were to determine the optimal conditions for clean-up of samples labeled with TMTpro reagents and to assess the efficiency of off-line high pH reversed-phase fractionation of these samples prior to LC-MS analysis. This was important to understand as the added hydrophobicity imparted by the more hydrophobic TMTpro reagent tag may significantly affect the effectiveness of a clean-up protocol optimized for the removal of the TMT reagents. Likewise, this change in hydrophobicity can have a significant effect on off-line high pH reversed-phase fractionation profiles as well as the liquid chromatography in LC-MS analysis.

MATERIALS AND METHODS

HeLa S3 cells were grown in sMEM supplemented with 10% FBS, 1X Glutamax and 1% Pen/Strep. Starting input protein concentration was measured using Thermo Scientific™ Pierce™ Rapid Gold BCA Assay kit. Cell samples were processed using the Thermo Scientific™ EasyPep™ Maxi MS Sample Prep Kit to obtain ~1 mg of clean, MS-ready peptides. The yields were determined using the Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay. Single 100µg aliquotes of this sample were labeled with Thermo Scientific™ Tandem Mass Tag™ (TMT™) TMTzero reagent, Thermo Scientific™ TMTpro™ TMTpro-zero reagent, or left untreated (for label-free analysis). Fractionation of the peptide samples was performed using the Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit.

Protein digest samples (1µg per injection) were analyzed using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system using a 50cm C18 Thermo Scientific™ EASY-Spray™ column coupled to Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. LC gradient is shown in Figure 2.

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), deamidation (N, Q), and TMTzero or TMTpro-zero (where appropriate). Data were searched against the Uniprot human protein database and results were filtered using a 1% protein FDR threshold.

To evaluate the TMTpro reagent clean-up efficiency, 1mg of TMTpro-zero reagent was fully hydrolyzed by dissolving in 100µL of water and incubating for 24 hours at 50ºC; another 1mg of TMTpro-zero reagent was fully quenched with hydroxylamine by dissolving in 100µL of 10% aqueous hydroxylamine solution and incubating for 1 hour. Completion of the reactions were assessed by direct infusion MS. Following this, the two samples were mixed, acidified with trifluoroacetic acid, applied onto a conditioned high pH reversed-phase fractionation column, and subjected to fractionation according the step-gradient defined in Figure 1. Relative abundances of the two forms of TMTpro-zero reagent in each collected fraction were measured by direct infusion MS.

RESULTS

Figure 1. Removal of Excess TMTpro Reagent by High pH Reversed-Phase Off-Line Clean-up.

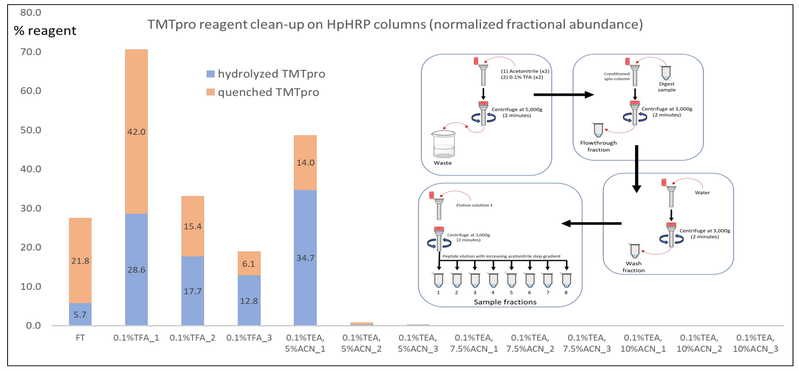
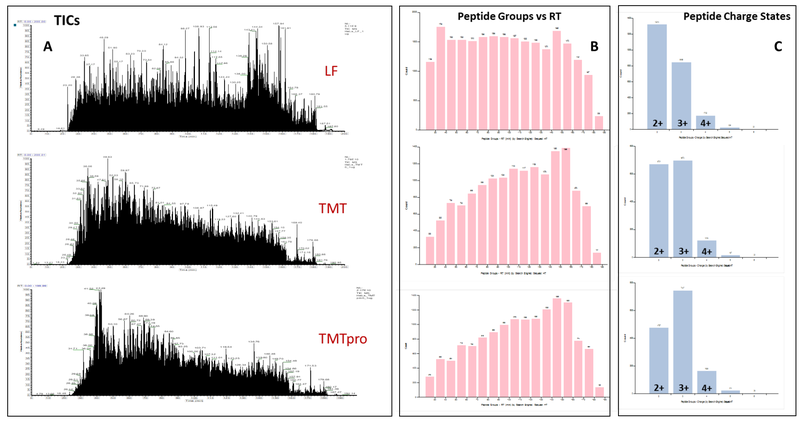


Figure 2 . LC-MS of unlabeled, TMT- and TMTpro- labeled HeLa digests



The same LC gradient was applied in the analysis of the unlabeled (LF), TMT-labeled, and TMTpro-labeled samples to assess the trends in peptide elution of the unfractionated samples. Figure 2A clearly shows the need for further LC gradient optimization for the analysis of the labeled samples, as the peptide identifications appear to be bunched towards the higher retention time in the gradient. Figure 2B & 2C show that PSM/peptide charge states increase with the increasing size of the peptides (LF<TMT<TMTpro peptides). Overall, both high pH reversed phase fractional resolution and retention of TMTpro-labeled samples was nearly the same compared to TMT-labeled samples (Figure 3-5)

Figure 3. Fractional Resolution of TMT and TMTpro Reagent-Labeled Samples.

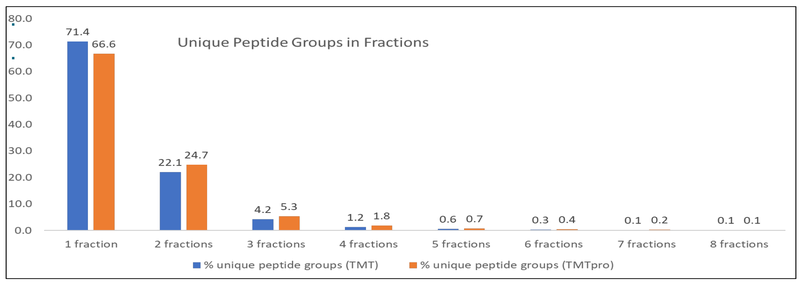


Figure 4. High pH Reversed-Phase Fractional Profiles Assessed by Different Methods.

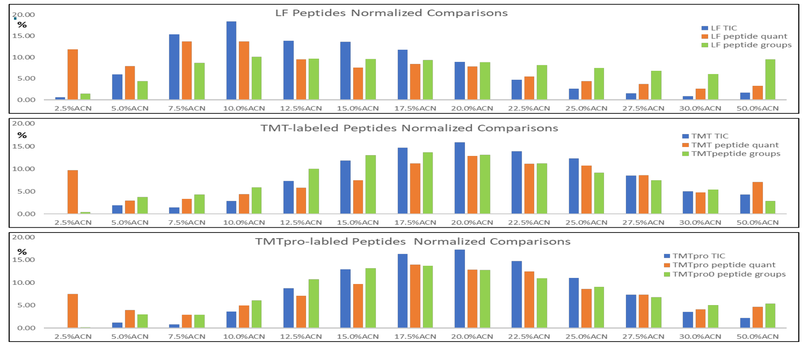
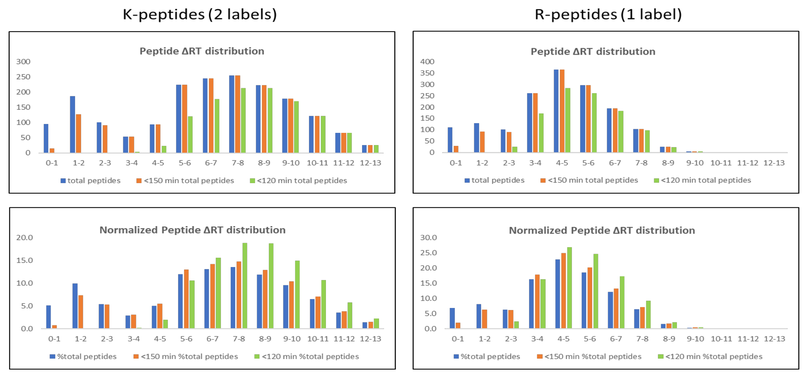


Figure 5. Distribution of Retention Time Differences for TMTpro and TMT-labeled Peptides.



CONCLUSIONS

- TMTpro-labeled peptide samples can be efficiently cleaned-up to remove the excess reagent and fractionated using a high pH reversed-phase resin with similar wash and step-gradient conditions previously optimized for TMT-labeled samples.
- TMTpro-labeled peptides are more hydrophobic than their TMT-labeled analogues and experience on-column retention shift on reversed-phase resins at low pH, but have similar retention at high pH.
- Step-gradients currently recommended for off-line clean-up and high pH reversed-phased fractionation may be successfully applied to samples labeled with TMTpro reagents to achieve similar fractionation efficiency.
- High pH reversed-phase fractionation of samples is required to extract the most protein identifications and protein quantitation information from a given proteomics sample.

TRADEMARKS/LICENSING

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