Analysis of Subtle Changes in Biological Systems Through use of High Resolution, High Accuracy UHPLC Generated Libraries with a Q-Exactive HF Mass Spectrometer

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Overview

Purpose: A robust method for discovering subtle changes in protein expression in biological systems.

Methods: Using AMAT(Accurate Mass, Accurate Time) libraries of peptides generated from both fractionated and unfractionated trypsin digests of protein lysates and biological fluids.

Results: Subtle expression differences in both plasma and cell lysates from the model system are revealed using the workflow described.

Introduction

Protein diversity in biological systems is quite large, but short term (<1hour) changes due to a particular stimulus will cause only subtle changes in very specific proteins expression levels. Excessive sample handling/processing methods often add significant noise to overall experimental results. Here we use a combination of simple sample preparation technique, extremely reproducible UHPLC system, and a robust mass spectrometer platform to afford researchers the ability to use massive protein/peptide libraries for quantification of several thousand proteins.

To study the effectiveness of this method, a model system using whole live bacteria, both gram negative and positive strains, were spiked into whole blood samples from a single donor. After 1 hour, plasma and peripheral blood mononuclear cells were isolated and analyzed for protein expression differences.

The Thermo Scientific™ Vanquish™ UHPLC platform with a tandem column setup allows for extremely high reproducibility across a wide range of sample loadings, independent of sample/matrix type. This allows the use of upfront sample fractionation to reduce peptide complexity and hybrid tandem mass spectrums for more confident and complete library generation, without compromising accurate retention time information.

Methods

Sample Preparation

Lithium heparin collected blood in plasma tubes, and Cell Preparation Tubes from an anonymous donor was spiked with buffer, 50 CFU/mL of E. coli, or 50 CFU/mL S. Aurus and allowed to incubate for 1 hour at 37 °C. The samples were then spun at 2500 RCF for the plasma separation or at 1800 RCF for Peripheral Blood Mononuclear Cell (PBMC) preparations. Both the plasma and cells were diluted and lysed in 8M GuHCl 250mM Tris-HCl 5%n-propanol 10mM DTT(pH 8.5). Cysteines were alkylated with the addition of iodoacetic acid to 45mM. Samples were diluted in digestion buffer, 50mM Tris-HCl 5mM CaCl2, and Pierce sequencing grade modified trypsin was added. Samples were digested overnight.

Liquid Chromatography

Peptide libraries were made by fractionating the peptides from both the plasma and cell lysate samples into 24 fractions on a 4.6mmx250cm PSDVB column 300A pore, 8um particle on a gradient from 99% 50mM ammonium acetate 0.4% ammonium hydroxide/water to 45% 0.2% ammonium hydroxide 10% water/acetonitrile.

UHPLC was setup with three Thermo Scientific™ Acclaim™ 120 C18 3µm 120A 2.1mmx250mm columns connected by Thermo Scientific™ Dionex™ Viper™ Fingertight Fitting tubing; both the solvent pre-heater and the column compartment were set to 55 °C. Solvent A was 0.2 formic acid in Fisher Optima LC-MS grade water, Solvent B was 0.2 formic acid in Fisher Scientific™ Optima™ LC-MS grade acetonitrile. A gradient of 3-50% over 95minutes at 250 µL/min with a total run length of 130 min. A divert valve diverted the solvent for the first 10 minutes and the last 20 minutes of the gradient. Pierce Retention Time Calibration standards are added to 1 pmole per injection.

Mass Spectrometry

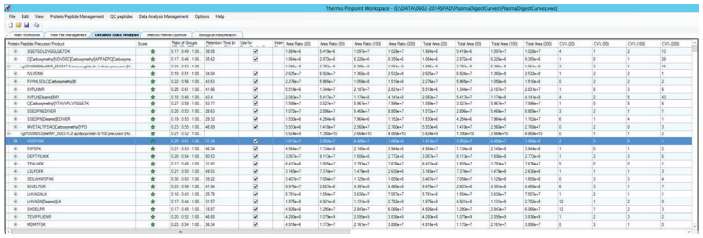
A Thermo Scientific™ Q Exactive™ HF Mass Spectrometer equipped with a HESI-II source with the spray cone installed was tuned with 35 sheath, 8 aux, 1 sweep with a vaporizer temperature of 275 °C and a capillary temp of 325 °C. The instrument acquisition is configured with a data dependant “top 20” method with 350-1500m/z on the full scan 3e6 target 120K resolution, ms/ms are triggered on apex triggers 6-20s peak width, 2e4 target(100%), 50ms fill, 15k resolution. Dynamic exclusion set to 20s, 25ppm.

Data Analysis

Raw files are searched Proteome Discoverer 1.4 using a simple Sequest search template. Parent mass tolerances were set to 5ppm, and fragment mass tolerances were set to 15mmu. Plasma and cell lysate data was searched with static carboxymethyl modified cysteines and differential oxidized methionines. Cell lysate data was searched with additional phosphorylations on serines, threonines, and tyrosines with PhosphoRS3.0 confirmation node set to 0.015 fragment ion tolerance. Result files(MSF) were imported into PinPoint for quantification.

FIGURE 1. Vanquish UHPLC system and Q Exactive HF MS.

A



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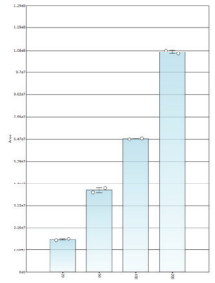

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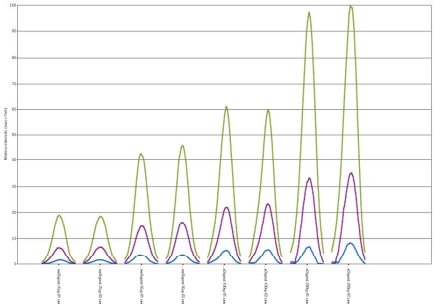


FIGURE 2. System loading testing, showing the robustness of quantification and retention time reproducibility 20-200ug loading of plasma peptides on column. A) global protein comparison in PinPoint; B) area and SD comparison for replicate injections C) isotope area comparison.

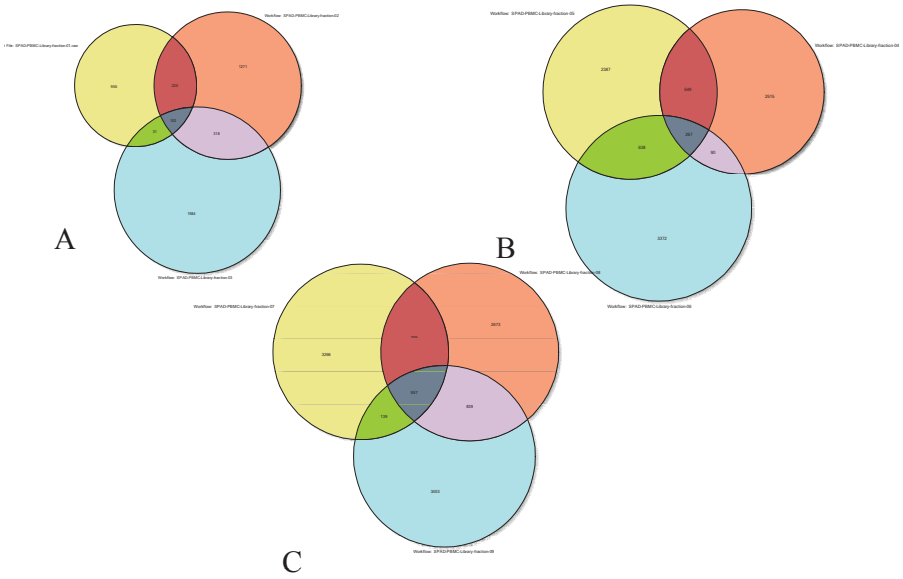
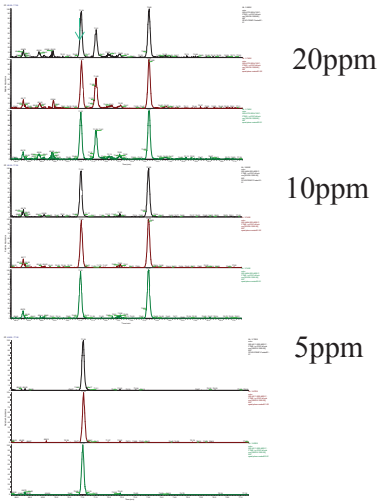


FIGURE 3. Unique peptides identified in sequential high pH reverse phase fractions A)1-3 B) 4-6 C)7-9.

Library Generation

In **Figure 2** we show that the peptide accuracy and quantification is stable over a large loading range. This is important as library fractions will have high concentrations of abundant peptides. In **Figure 3** we show that the fractionation is quite efficient, with minimal peptide overlap between fractions.

FIGURE 4. Even with high resolution accurate mass spectrometry, retention time accuracy is important to assign masses to library members that may not be intense enough to trigger MS2 events. Three different samples compared at 3 different mass filter tolerances in a 12 minute window. Peptide TEFLSFMNTELAAFTK, S100, A11 protein.



Results

Whole Blood Stimulation

Plasma library generation yielded 790 plasma protein groups (1% FDR) 6067 peptides with +/-10s retention time accuracy.

PBMC library generation yielded 6200 Protein groups(1% FDR) 53,444 peptides, 1072 phosphopeptides(no specific enrichment)

As it would be a technical challenge to quantify ALL the proteins in either one of these samples, selected protein and peptides from each sample can be selected from the library.

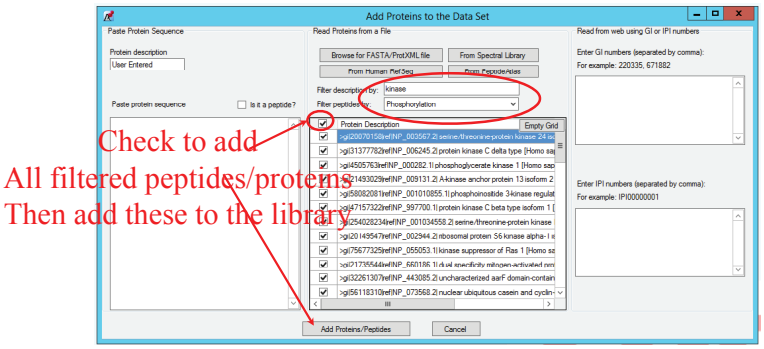


FIGURE 5. PinPoint library selection tool allows users to select peptides by mods or proteins by name.

Comparison of Proteins in Plasma and PBMC samples

Selected proteins from plasma and PBMC libraries were compared in both the plasma and PBMC samples. This allowed for direct quantification between the two sample types as well as across the stimulation conditions. The S100 protein class was chosen as it is well represented in infection models and circulating immune cells.

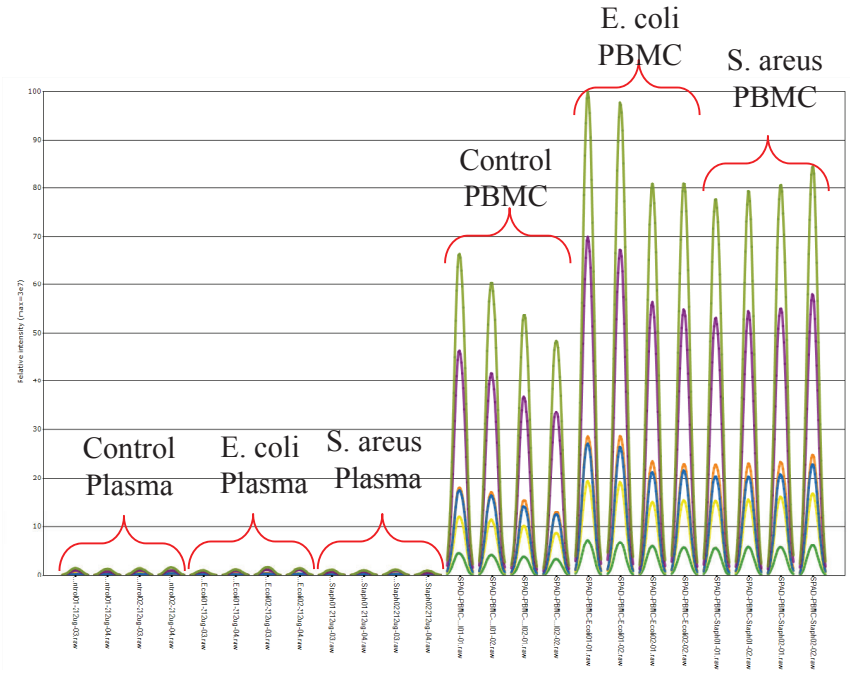


FIGURE 6. S100 Calcium binding protein-A8 Calprotectin in plasma and PBMC lysates showing top 3 isotope XICs for +2 and +3 charge states, 2 technical and 2 experimental replicates per sample state.

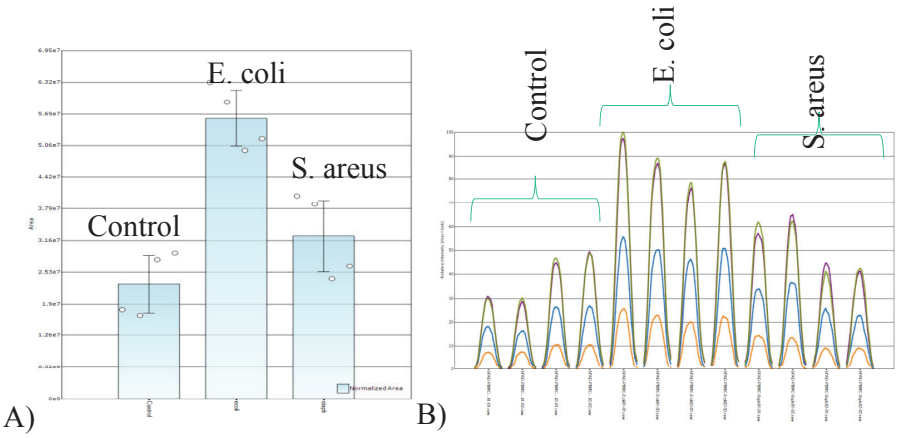


FIGURE 7. Phosphopeptide FYAAEIASALGYLHSIK **from Serine/Threonine protein kinase SGK isoform1 from PBMC lysates A) area sum of peptides B) peptide isotope quantification.**

High dynamic range quantification

Even without targeted quantification methods, low level phosphopeptides can be analyzed and quantified. The variability of phosphorylation amongst experimental replicates is a function of the modification's biological variability in the system used, even with the highly simplified sample preparation techniques used. This new workflow affords researcher a facile method for the rapid qualitative and quantitative analysis of complex biological systems.

Conclusion

- Three months of operation on one column setup with sub 4 second shift in retention time allows for libraries and samples to be run continuously.
- The only cleaning performed was the swapping of the heated ion transfer capillary, and washing off the sweep cone after each sample set/type.
- Raw digests with no cleanup additional sample preparation yielded no increase in column pressures.
- Extremely reproducible injections allows for measurements of small biological changes, including low level modified peptides without going to targeted methods.

References

1. Hansen, W. L. J.; Bruggeman, C. A.; Wolffs, P. F. G. Evaluation of New Preanalysis Sample Treatment Tools and DNA Isolation Protocols To Improve Bacterial Pathogen Detection in Whole Blood. *Clin. Microbiol.* **2009**, 47, 8, 2629—2631 http://jcm.asm.org/content/47/8/2629.full

2. Hao, P.; Ren, Y.; Dutta, B.; Sze, SK. Comparative Evaluation of Electrostatic Repulsion–Hydrophilic Interaction Chromatography (ERLIC) and High-pH Reversed Phase (Hp-RP) Chromatography in Profiling of Rat Kidney Proteome. *Journal of Proteomics*, **2013**, 82, 254—262

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