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Abundant Protein Depletion of Human Plasma Samples – A Reproducibility and Scaling Study

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ABSTRACT

Introduction: The large dynamic range in protein abundance of plasma samples is the main problem associated with plasma/serum-based biomarker discovery experiments, and depletion of abundant proteins is required in order to identify and measure changes in prognostic or diagnostic plasma proteins. We have optimized the production and immobilization of immunoaffinity ligands to develop new top2 and top12 abundant protein depletion resins. In addition, we comprehensively evaluated of the specificity, efficiency, and reproducibility abundant protein depletion from human plasma samples

Methods: A label-free approach was used for relative quantitation of human plasma proteins by LC-MS. All samples were analyzed on Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer and processed using Thermo Scientific™ Proteome Discoverer™ 1.4 and Skyline 3.6 (University of Washington) software.

Summary: We have optimized the conjugation chemistries and blending protocols to achieve >95% depletion efficiency of target high abundance proteins in human plasma samples. We have assessed depletion capacity of the new depletion resins to define the practical range of sample amounts in terms of depletion efficiencies, post-depletion protein yields, and reproducibility.

Novel Aspects: Development and comprehensive application analysis of new top2 and top12 depletion resins for abundant human plasma proteins

INTRODUCTION

Reproducibility of proteins depletion is of paramount importance when profiling biological samples with protein concentrations covering a broad dynamic range. We have recently developed two new resin formulations, Top2 (Albumin, IgGs) and Top12 (Albumin, IgGs, transferrin, fibrinogen, alpha-1 antitrypsin, apolipoprotein A1, alpha-2 macroglobulin, alpha-1 acid glycoprotein, haptoglobin), to be used for depletion of abundant human plasma proteins in a disposable spin column format. The new columns were designed to accommodate two sample sizes, 10µL and 100µL of plasma, and all bind/wash/elute conditions were optimized to yield maximum depletion of the targeted proteins and maximum recovery of the non-targeted proteins, to minimize the number of handling steps and processing time, and to maintain excellent sample-to-sample reproducibility in depletion and recovery. In this study, we focused on assessing reproducibility in terms of (a) total protein material depletion, (b) specific depletion of targeted proteins, and (c) abundance of non-targeted proteins post-depletion.

MATERIALS AND METHODS

Normal pooled plasma from a commercial source was used in this study. Briefly, columns were allowed to equilibrate columns to room temperature,10uL of human plasma were added to the column, which was then capped and vortexed gently back into suspension. The column was then placed on rotator to mix. After 30 minutes of incubation, the column was centrifuged at 1500 x g for 2 minutes and the flow through fraction was collected into a clean 2mL microcentrifuge tube. The Top2 and the Top12 flow through was in a final volume of 110uL and 210uL, respectively. Triplicate samples were generated for each Top2 and Top12 depletions and protein recovery was measured using BCA assay (Thermo Fisher Scientific, P/N 23225) and compared to the total protein content of undepleted plasma.

The samples were dried and resuspened in 200 uL of 3% SDS, 100 mM HEPES (pH 8.5) containing 50 mM tris(2-carboxyethyl)phosphine (TCEP) and 100 mM chloroacetamide (CAA). The samples were reduced and alkylated by heating the mixture at 95 ºC for 5 minutes. Proteins were precipitated from acidified methanol, protein pellet was washed several times with neat methanol and dissolved in 200 uL of 25 mM HEPES (pH 8.5) by sonication in a water bath. MS-grade trypsin (Thermo Fisher Scientific, P/N 90058) was added in a 1:50 enzyme-to-substrate ratio and the samples were incubated at 40 ºC for 16 hours. After digestion, the samples were desalted and peptide recoveries were measured using Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific, P/N 23275) and were compared to the peptide content of the undepleted samples, which were prepared in parallel as controls.

Figure 1. Total plasma protein removal after Top2 and Top12 depletion

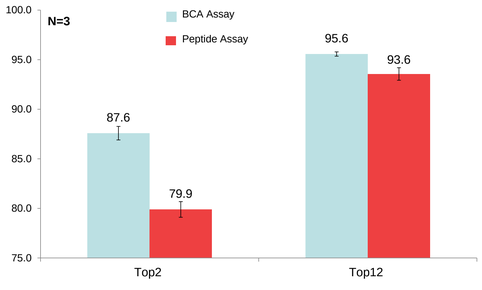
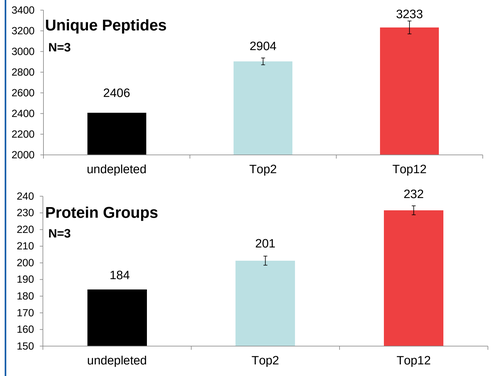


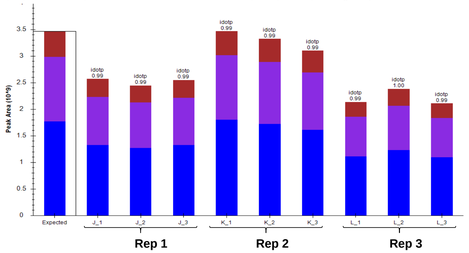
Figure 2. Unique Peptide and Protein Group Identifications After Depletion



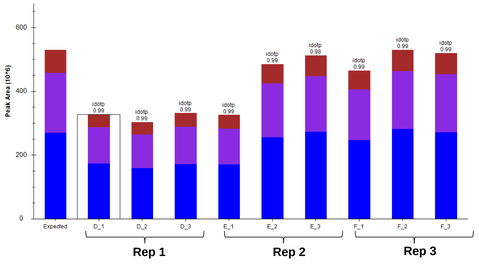
The samples were analyzed by LC-MS by performing triplicate injections of ~750 ng (for each prepared sample replicate). All samples were run on a the Orbitrap Fusion Tribrid mass spectrometer. Liquid chromatography was performed using Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system, utilizing a 50 cm C_{18} Thermo Scientific™ EASY-Spray™ column heated at 45 ºC. Raw data was processed using Thermo Scientific™ Proteome Discoverer™ 1.4 and Skyline 3.6 (University of Washington) software.

Figure 3. Reproducibility in Quantitation of a Selected Peptide After Top2 and Top12 Depletion.

Apolipoprotein A1 peptide K.DLATVYVDVLK.D after Top2 depletion (not targeted)



Apolipoprotein A1 peptide K.DLATVYVDVLK.D after Top12 depletion (targeted)

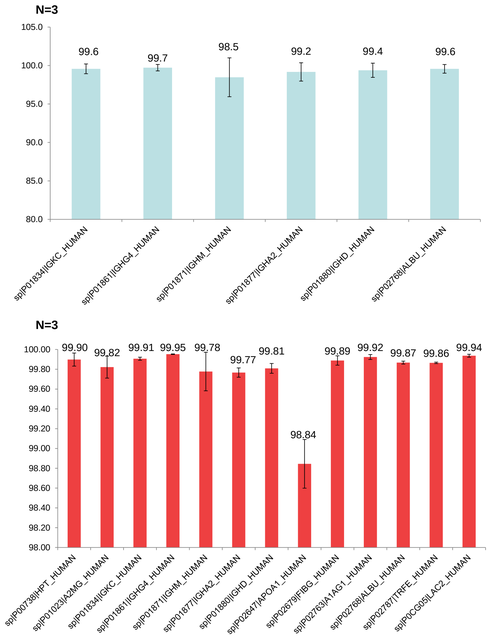


RESULTS

After Top2 and Top12 depletion was performed on 10 uL of human plasma, amount of protein material recovered was measured at the protein level using BCA assay and at the peptide level using Pierce Quantitative Colorimetric Peptide Assay. Percent depletion of total protein material was calculated and the protein/peptide level results were compared. The results show good agreement with low %CV within each replicate set (Figure 1). This information can be used to establish what fraction of the total recovered sample can be taken for LC-MS analysis to maximize the identification numbres without overloading the column.

LC-MS data from triplicate injections of 750 ng of each replicate sample was processed (high confidence peptides; minimum 2 unique peptides per protein) and identification numberrs were compared (Figure 2). After Top2 and Top12 depletion, we obtained 10% and 30%, respectively, increases in protein group identification numbers relative to the undepleted samples.

Figure 4. MS-based assessment of abundant protein depletion.



To assess the percent depletion for individual targeted proteins, 2 to 8 unique, well-behaved peptides were selected for each target protein. Their abundance (peak area) was monitored at MS-level by accurate mass and retention time. It is important to establish reproducibility not only in depletion of the targeted proteins, but also in the recovery of non-targeted proteins. Figure 3 shows peak areas of a selected peptide derived from apolipoprotein A1. Our data suggests that reproducible recovery of the targeted, as well as the non-targeted proteins, is obtained following the depletion, with the CVs of less than 30% (for both the injection replicates and the biological replicates).

Depletion percentages for each targeted protein in the Top2 and Top12 depletion were calculated (Figure 4). Using average sums of peak areas of all “proteotypic” peptides for triplicate injection replicates. For all targets, >95% depletion was attained. Excellent reproducibility was observed for each set of samples, with less than 1% CVs.

Depletion numbers from the MS-based analysis were compared to those generated with ELISA (Table 1). The numbers generated by these two techniques were in good agreement.

Table 1. Assessment of abundant protein depletion by ELISA.

ELISA RESULTS	Top2	Top12
Albumin	99.2	99.6
IgG Kappa	99.1	99.9
IgG Lambda	99.0	99.9
Acidglycoprotein	NA	99.3
Antitrypsin		100
Macroglobulin		99.9
Apolipoprotein		97.4
Fibrinogen		99.8
Haptoglobin		99.4
Transferrin		99.8

CONCLUSIONS

- Reproducible depletion of abundant proteins is obtained by using Pierce™ Top2 and Top12 abundant protein depletion spin columns.
- Abundant protein depletion from plasma allows for detection of more proteins in the sample enabling better detection and quantitation of relevant biomarkers.
- While reproducible depletion of the abundant proteins can be attained, MS-based label-free quantitation ultimately depends on the quality of sample preparation after the depletion, in terms of reproducibility of reduction/alkylation, digestion efficiency and peptide recovery.

TRADEMARKS/LICENSING

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