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Comparative evaluation of seven commercial products for human serum enrichment/depletion by shotgun proteomics

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ABSTRACT

Seven commercial products for human serum depletion/enrichment were tested and compared by shotgun proteomics. Methods were based on four different capturing agents: antibodies (Qproteome Albumin/IgG Depletion kit, ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit, Top 2 Abundant Protein Depletion Spin Columns, and Top 12 Abundant Protein Depletion Spin Columns), specific ligands (Albumin/IgG Removal), mixture of antibodies and ligands (Albumin and IgG Depletion SpinTrap), and combinatorial peptide ligand libraries (ProteoMiner beads), respectively. All procedures, to a greater or lesser extent, allowed an increase of identified proteins. ProteoMiner beads provided the highest number of proteins; Albumin and IgG Depletion SpinTrap and ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit resulted the most efficient in albumin removal; Top 2 and Top 12 Abundant Protein Depletion Spin Columns decreased the overall immunoglobulin levels more than other procedures, whereas specifically gamma immunoglobulins were mostly removed by Albumin and IgG Depletion SpinTrap, ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit, and Top 2 Abundant Protein Depletion Spin Columns. Albumin/IgG Removal, a resin bound to a mixture of protein A and Cibacron Blue, behaved less efficiently than the other products.

1. Introduction

One of the main goals of Mass Spectrometry (MS)-based proteomics is biomarker discovery. The high resolution, sensitivity and accuracy offered by current instruments allow to obtain reliable spectra and robust identifications and to explore many orders of dynamic range of protein concentration in complex samples [1]. The most investigated clinical specimens are biofluids, like serum, plasma and urine, due to their availability by minimally or non-invasive procedures, as well as their function as metabolites reservoirs [2–5]. However, the whole proteome coverage for such complex biological samples is still unachievable due to the presence of high concentration of few proteins. In particular, albumin, immunoglobulins, serotransferrin and haptoglobin represent more than 99% of the total serum and plasma proteins and interfere with the detection of less abundant proteins, limiting the analytical efficiency of LC-MS/MS [6,7]. Therefore, their removal is essential to make low-abundant species detectable, including physiologically important molecules such as cytokines, chemokines, lipoproteins and peptide hormones, that represent the most valuable source of disease biomarkers. To this extent, one of the most challenging tasks in biomarker discovery studies is reducing the huge dynamic range of protein concentration, which takes approximately 10 orders of magnitude in serum. Many protein depletion strategies have been attempted on blood serum and other biofluids to decrease the concentration of the most abundant proteins before MS analysis. Specifically, methods based on ultrafiltration, precipitation and differential extraction with appropriate chemical reagents, combinatorial peptide ligand libraries, magnetic nanoparticles, and commercial depletion kits based on affinity and/or immunoaffinity have been proposed [2,4,6,8–16]. Aim of this work is to provide an exhaustive and easily reproducible comparison of the pre-analytical variables obtained with a wide selection of commercially available kits. Hence, seven products were selected according to their highest throughput, given by the use of spin columns devices, and different technologies. Four of them are based on immunoaffinity, with antibodies directed against specific proteins [11–14]; one is a mixture of protein G and antibodies directed against human serum albumin [15]; another one is composed by a mixture of protein A and a dye-ligand widely recognized as Cibacron Blue [16], and the last is based on the affinity for a combinatorial peptide ligand library [10]. All products were applied to the same commercial standard human serum

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and the enriched proteins were evaluated by SDS-PAGE, high resolution shotgun MS analysis and label-free quantitation.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were LC or electrophoresis grade. Dithiothreitol (DTT), iodoacetamide (IAA), β-mercaptoethanol, glycerol, sodium dodecyl sulfate (SDS), Tris-base, glycine, bromophenol blue, urea, ammonium bicarbonate (ABC), trypsin, phosphate buffered saline (PBS) tabs and ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit (ProteoPrep) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Any kD Mini-PROTEAN TGX Stain-Free protein gels, Precision Plus protein electrophoresis standards and ProteoMiner beads (ProteoMiner) were acquired from Bio-Rad (Hercules, CA, USA). Albumin and IgG Depletion SpinTrap (SpinTrap) and Qproteome Albumin/IgG Depletion (Qproteome) kits were obtained from GE Healthcare (Uppsala, Sweden) and Qiagen (Sollentuna, Sweden), respectively. Top 2 Abundant Protein Depletion Spin Columns (Top 2), Top 12 Abundant Protein Depletion Spin Columns (Top 12), Albumin/IgG Removal (CibB-A), BCA protein assay kit and SimplyBlue safestain were acquired from Thermo Fisher Scientific (San Jose, CA, USA). Amicon Ultra Centrifugal filters were from Merck Millipore, (Burlington, MA, USA). Acetonitrile and formic acid were obtained from Carlo Erba (Val-de-Reuil, France). MassPREP *E. Coli* Digest Standard were from Waters (Milford, MA, USA). Serum sample (ECHS Working Standard Human Serum) was purchased from NIBSC (Potters Bar, UK).

2.2. Serum sample

The lyophilized serum sample was reconstituted at a protein concentration of 87 mg/mL with Milli Q water and stored at − 80 °C until use.

2.3. Sample treatment

2.3.1. Pierce Top 2 and Top 12 Abundant Protein Depletion Spin Columns

Depletions were performed according to the manufacturer's recommendations. Briefly, 10 µL of serum were applied to each column and incubated, with gentle end-over-end mixing, for 30 min (Top 2) or 60 min (Top 12). Depleted flow throughs were recovered by centrifugation at 1000 × *g*.

2.3.2. Sigma-Aldrich ProteoPrep Immunoaffinity Albumin and IgG Depletion Kit

Depletion was performed according to the manufacturer's recommendations. Briefly, 25 µL of sample were diluted to 100 µL with equilibration buffer supplied in the kit. Samples were applied to a pre-equilibrated column and, after an incubation of 10 min, flow through was collected by centrifugation at 8000 × *g* and reapplied to the column for 10 min. Column was washed with 125 µL of equilibration buffer and the collected filtrate was mixed with the flow through.

2.3.3. GE Healthcare Albumin and IgG Depletion SpinTrap and Qiagen Qproteome Albumin/IgG Depletion kit

Depletion was performed according to the manufacturer's recommendations. Briefly, 25 µL of serum were diluted to 100 µL with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (equilibration buffer), applied to each pre-equilibrated column and incubated for 5 min. Depleted flow throughs were then recovered by centrifugation at 800 × *g*. Columns were washed twice with 100 µL of equilibration buffer and the collected filtrates were mixed with the corresponding flow throughs.

2.3.4. Pierce Albumin/IgG Removal Kit

Depletion was performed according to the manufacturer's recommendations. Briefly 10 µL of serum were diluted to 75 µL with 25 mM Tris-HCl, 25 mM NaCl, 0.01% NaN_3, pH 7.5 (equilibration/washing buffer) supplied in the kit, applied to 85 µL of dry resin and incubated in a Thermomixer (Eppendorf, Hamburg, Germany) for 10 min at 450 rpm. Flow through was collected by centrifugation at 10,000 × *g* and reapplied to the column for 10 min. Column was washed with 75 µL of buffer and the collected filtrate was mixed with the flow through.

2.3.5. Bio-Rad ProteoMiner Protein Enrichment beads

Enrichment was performed according to the manufacturer's instruction, with minor modifications. Briefly, 40 µL of serum were applied to 4 µL of dry beads previously equilibrated with 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (equilibrating/wash buffer), and incubated in a rotating shaker for 2 h, RT. After discarding flow through, beads were washed three times. Bound proteins were eluted by incubating the beads with 100 mM Tris-HCl, 0.8%. SDS, pH 6.8. Two consecutive elution steps were performed, the former by incubating the beads with 20 µL of buffer for 1 h on a rotating shaker, and the last with 40 µL of buffer for 15 min. Eluted fractions were pooled for further analysis.

2.4. Concentration, buffer exchange, quantification and SDS-PAGE of protein fractions

Depleted and enriched fractions collected from all the procedures were ultrafiltrated on Amicon Ultra centrifugal filters, 10 kDa cut off, in order to obtain homogeneous conditions for the quantification step. Each fraction was exchanged with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, and brought to a final volume of 50 µL. Protein concentration was evaluated by BCA protein assay and measurements were performed on a Magellan Sunrise Spectrophotometer (Tecan, Männedorf, Switzerland), at 560 nm. Finally, a non-reducing Laemmli buffer [17] was added to 10 µg of each depleted/enriched fraction, and subjected to SDS-PAGE on any kD acrylamide gels using the Mini-Protean system (Bio-Rad, Hercules, CA, USA).

2.5. Filter aided sample preparation (FASP)

Depleted/enriched sera were treated according to a modified "FASP II" protocol [18,19], with minor changes. Briefly, protein samples were diluted to 500 µL in 8 M urea in 0.1 M Tris-HCl pH 8.8 (UT), transferred to 10 kDa cut off Amicon Ultra centrifugal filters, and centrifuged at 12,000 × *g* for 15 min. Concentrates were then diluted in UT buffer and centrifuged again. Then, proteins were reduced in 10 mM DTT in UT at 20 °C, 600 rpm for 30 min, and then alkylated in 50 mM IAM in UT at 20 °C, 600 rpm for 20 min. After 5 washes (3 in UT and 2 in 50 mM ABC), trypsin solution was added to the filters (enzyme-to-protein ratio 1:100 w/w), and samples were incubated at 37 °C overnight. Peptides were collected by centrifugation followed by two additional washes of the membranes with 70% ACN, 1% formic acid. Finally, the peptide mixtures were brought to dryness and reconstituted in 0.2% formic acid. Peptide mixtures concentration was estimated by measuring the absorbance at 280 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, San Jose, CA, USA), using dilutions of the MassPREP *E. Coli* Digest Standard to generate a calibration curve.

2.6. LC-MS/MS analysis

Peptide mixtures were analyzed on a Q Exactive mass spectrometer interfaced with an UltiMate 3000 RSLCnanoLC system (Thermo Fisher Scientific, San Jose, CA, USA), as previously described [20] with some adjustments. A total of 4 µg of each peptide mixture was concentrated and washed on a trapping precolumn (Acclaim PepMap C18, 75 µm ×