Abstract

It is believed that the analysis of the serum proteome will provide access to new protein markers that may hold the information needed to revolutionize disease diagnosis and therapeutic monitoring. However, the masking effect of a few well-characterized, high-abundant proteins in serum has been a major obstacle for the detection of low-abundant proteins that may be of interest for biomarker identification. The Agilent Multiple Affinity Removal System combines the specificity of antibody-antigen recognition and the efficiency of standard liquid chromatography instrumentation for high-abundant protein removal. A ready-to-use high-abundant protein removal system, consisting of an affinity column and optimized mobile phases (Buffers A and B), is available for simultaneous removal of multiple proteins from human serum in a single step. The affinity column removes albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin with high specificity and reproducibility. The buffers are optimized to minimize removal of proteins not targeted by the immobilized antibodies. As a result, high-value, less-abundant proteins can be brought into detectable levels by loading up to 10 times more low-abundant protein mass onto gels for analysis than what was possible before depletion, as shown in this study.

Introduction

While playing a vital role in circulating important molecules throughout the body, human plasma has long been recognized as an important source for biomarkers associated with physiological conditions. A complete picture of the whole plasma proteome will provide further understanding of its dynamic changes associated with various disease states, which in turn would enable characterization of new markers and therapeutic targets. However, the concentrations of proteins found in human serum are estimated to span over 12 orders of magnitude. A few high-abundant proteins like albumin and immunoglobulin dominate the protein mass and mask the detection and identification of low-abundant biomarkers.

The Agilent Multiple Affinity Removal System simultaneously removes multiple high-abundant proteins from human plasma/serum in a single step. The Multiple Affinity Removal System is comprised of an affinity column and optimized mobile phases (Buffers A and B). The affinity column is packed with immobilized affinity-purified polyclonal antibodies for removal of albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin with high specificity. Therefore, while passing human serum or plasma through a single column, all six targeted proteins are captured and removed from the mixture simultaneously. Buffers A and B are optimized to minimize removal of proteins not targeted by the antibodies and to ensure reproducibility of column performance and long column lifetime. As a result, this technology enables the
expansion of the detectable dynamic range of 1D gel electrophoresis (1DGE) [1], 2D gel electrophoresis (2DGE), and liquid chromatography/mass spectrometry (LC/MS) by removing the interferences. We have processed human serum using this affinity system and demonstrated reproducible removal of target proteins using 1DGE [1]. In this application, 2DGE was used to resolve affinity-depleted serum proteins.

Experimental

Human sera and plasma were purchased from Sigma (St. Louis, MO). The Agilent Multiple Affinity Removal System for removal of albumin, transferrin, IgG, IgA, haptoglobin and antitrypsin from human serum was developed in-house at Agilent Technologies (Wilmington, DE). A 4.6 × 50 mm Multiple Affinity Removal column with binding capacity for 20 µL of human serum was used. A mobile phase reagent kit for the affinity column was used for sample loading, washing and column regeneration (Buffer A) and for bound protein elution (Buffer B) from the column.

HPLC Protocol for Immunoaffinity Chromatography

High-abundant protein removal from crude human serum was performed according to a standard LC protocol provided with the Agilent Multiple Affinity Removal System. Briefly, crude human serum and plasma samples were diluted five times with Buffer A containing protease inhibitors (COMPLETE™, Roche) and filtered through 0.22-µm spin filters by spinning at 16,000 × g at room temperature for 1–2 min. Automated sample injection was set for 75 µL of diluted plasma sample per injection in Buffer A (%B) at a flow rate of 0.25 mL/min for 9 min. The bound fractions were eluted with Buffer B (100% B) at a flow rate of 1.0 mL/min for 3.5 min. Then, the column was regenerated by equilibrating with Buffer A (0% B) for 7.5 min with a total run cycle of 20 min. Consecutive injections of a diluted human plasma sample were set for 200 runs under standard conditions with flow-through fractions collected (0.75 mL) at 1.5–4.5 min automatically into 1.5-mL plastic tubes (Sarstedt, Numbrecht, Germany). All chromatographic fractionations were performed at room temperature (22 °C) on an Agilent 1100 HPLC system with automated sample injector and fraction collector set at 4 °C. Collected flow-through fractions were stored at −20°C for further analysis.

Protein Concentrating with Spin Concentrators

To resolve depleted proteins on 2D gels, flow-through fractions from five injections were pooled into a spin concentrator with 5 kDa molecular weight cutoff (MWCO). The sample was spun at 7500 × g for 20 min at 10 °C. Then, buffer exchange was performed by three rounds of spinning of 4 mL of 10 mM Tris-HCl pH 7.4 through the spin concentrator under the same centrifugal conditions. Concentrated and buffer exchanged flow-through fractions were transferred into a fresh Eppendorf tube for storage. Protein content was estimated using Pierce BCA protein assay kit.

Results and Discussion

Target Multiple High-Abundant Proteins with Specific Antibodies

It is estimated that there is a broad dynamic range of protein amounts (10^{12}) in the human plasma proteome. When crude plasma or serum is resolved by current protein separation technologies such as 2DGE or MD-HPLC, only an incomplete picture - proteins present in the top 10^4 of dynamic range - can be detected. Figure 1 illustrates the Coomassie Blue stained 2D gel of normal human serum. The six most highly abundant proteins, indicated by arrows and the circled areas in Figure 1, represent approximately 85%–90% of total protein mass in the serum. They block the detection of low-abundant proteins by limiting the mass loading of other proteins. The Agilent Multiple Affinity Removal System was designed to target the six high-abundant proteins for removal. This system
uses affinity-purified polyclonal antibodies in an LC column format. The ratio of immobilized antibodies packed in the affinity column mirrors the amount of target proteins found in a given volume of serum samples. When a sample of serum/plasma is passed through the affinity column, all six target proteins in the sample are retained while all other proteins flow through and are separated from the high-abundant proteins.

Figure 1. Human serum protein pattern in a Coomassie Blue stained 2-D gel. First dimension IPG strips (pH 3–10 nonlinear, 11 cm) for 35,000 Vh. Second dimension was a vertical gradient slab gel (8%–16%). Sample loading: 250 µg of human serum proteins. Albumin, IgG, IgA, haptoglobin, transferrin, and antitrypsin are indicated by arrows and circled areas. The six high-abundant proteins are targeted for removal by the Multiple Affinity Removal System.
Remove Multiple-Targeted Proteins Simultaneously

Figure 2 shows a representative chromatogram of protein depletion from human plasma on a 4.6 × 50 mm Multiple Affinity Removal column. Buffer A (a neutral salt solution with phosphate at pH 7.4) is used for sample loading and column regeneration and Buffer B (a concentrated urea solution at pH 2.2) for stripping bound proteins. A low flow rate (0.25 mL/min) was used to ensure specific binding of targeted proteins and a higher flow rate (1.0 mL/min) was used to elute bound proteins. These conditions were developed to ensure run-to-run reproducibility, column regeneration, and reusability. Given the potential of coagulation in human plasma, fractionations of plasma with the Multiple Affinity Removal System were performed with and without EDTA in Buffer A and no differences or clogging were observed. Therefore, Buffer A was used, as supplied for the rest of the fractionations in this study, for multiple high-abundant protein removal from human plasma samples. As observed in the chromatograms for human serum [1], chromatographic consistency over 200 runs with consecutive injections of human plasma is shown in Figure 2, indicating that the affinity column performs with expected efficiency and reproducibility.

Bring Low-Abundant Proteins into Detectable Levels for Biomarker Identification

Flow-through fractions of human serum and plasma were collected and concentrated as described in the experimental section. Two hundred fifty micrograms of proteins were loaded for 2D gel electrophoresis. Figure 3 shows the protein pattern of human serum (3A) and plasma (3B) after immunodepletion with the Multiple Affinity Removal System as resolved on 2D gels. Circles indicate the areas where the target high-abundant proteins were removed by the Multiple Affinity Removal System. In the absence of the six high-abundant proteins, low-abundant proteins previously masked became detectable due to a 10-fold increase in mass loading of low-abundant proteins on the gel.

Figure 2. Chromatogram of the affinity removal of high-abundant proteins from human plasma. Consecutive injections of 75 µL of 5x diluted human plasma in Buffer A were made on a 4.6 × 50 mm Multiple Affinity Removal Column at a flow rate of 0.25 mL/min in Buffer A. Depleted protein fractions (flow-through) were collected from 1.5–4.5 min. The bound fraction was eluted with Buffer B at a flow rate of 1.0 mL/min for 3.5 min. Identical chromatograms were obtained when overlaying runs number 20 and 200 on an Agilent 1100 LC System. This result indicates high chromatographic reproducibility over 200 runs.
Figure 3. Coomassie Blue stained 2D gels. Protein pattern of human serum (3A) and plasma (3B) after immunodepletion using the Multiple Affinity Removal System. The circles indicate positions where the targeted high-abundant proteins were removed by the affinity column. First dimension: pH 3–10, nonlinear IPG strips (11 cm) for 35,000 Vh. Second dimension: vertical gradient slab gels (8%–16%). Sample loading: 250 µg of human serum proteins. Molecular weight markers: Mark12, (Invitrogen).
A Ready-To-Use Affinity Removal Kit

Results in this study demonstrate that the Multiple Affinity Removal System removes targeted proteins with a single affinity column of high reproducibility. The ready-to-use buffers ensure high column performance and reproducibility. More importantly, they allow comparison of experimental results from different laboratories using the Multiple Affinity Removal System for serum protein fractionations. Other components in the starter reagent kit provide convenient tools for sample dilution, filtration, and concentration, covering the entire workflow when using the affinity column. With an automated sampler and collector, removal of high-abundant proteins from human fluids such as serum, plasma, and cerebrospinal fluid (CSF) is simplified.

Enable Biomarker Identification

One goal of proteomics research is to identify biomarkers based on differences in protein levels and/or post-translational modifications. Initial sample fractionation helps reduce sample complexity and enables identification of markers at lower abundance. When selecting sample fractionation techniques, specificity and reproducibility are the key concerns. The Agilent Multiple Affinity Removal System uses a single affinity column to remove six targeted high-abundant proteins with high specificity and reproducibility. Columns can be reused for at least 200 injections without loss of binding capacity. The Agilent Multiple Affinity Removal System possesses the required features as the method of choice for removal of multiple high-abundant proteins for proteomics sample preparation. The depleted proteins are ultimately enriched and resolved with improved visibility using popular separation methodologies such as 1DGE, 2DGE, and LC/MS.

Reference


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