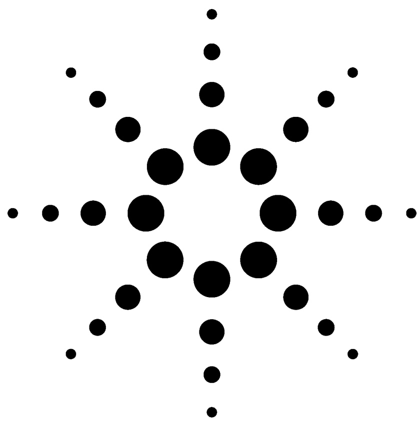

Agilent Multiple Affinity Removal System for the Depletion of High-Abundant Proteins from Human Serum - A New Technology from Agilent

Application

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Abstract

A novel Agilent Multiple Affinity Removal System has been introduced for depletion of six high-abundant proteins from human biological fluids. The system comprises an affinity column and optimized buffer reagent kit for reproducible column performance (at least 200 times) without loss of binding capacity. The affinity column removes six high-abundant proteins: albumin, IgG, IgA, haptoglobin, transferrin, and antitrypsin using affinity-purified polyclonal antibodies packed into an LC column. In this study, the affinity column and kit were used to remove the targeted high-abundant proteins from human serum under standard conditions. In the absence of the six high-abundant proteins (85%–90% of total protein mass in human serum), low-abundant proteins, recovered from flow-through fractions, were resolved on SDS-PAGE with ten times increase in mass loading, showing improvements in the number of identifiable proteins in human serum. In these studies, the multiple antibody-based affinity removal system proved to be a powerful tool for rapid, reliable, and specific removal of six high-abundant proteins in a single step.

Introduction

The rapid increase in the power of protein identification using LC/MS/MS and MALDI-MS has enabled proteomics scientists to tackle complex protein expression profiles on a large scale including the human plasma proteome. However, the masking effect of a few well-characterized, high-abundant proteins in plasma or serum has been a major obstacle for the detection of low-abundant proteins that may be of interest for biomarker identification. Current separation methodologies, such as one-dimensional gel electrophoresis (1DGE), two-dimensional gel electrophoresis (2DGE), and multidimensional high performance liquid chromatography (MD-HPLC) have a limited dynamic range for protein mass loading and detection. Removal of these high-abundance proteins would, therefore, allow a significant increase in relative mass loading of low-abundant proteins, expanding the dynamic range of these analytical methods. Removal of individual high-abundant proteins, such as albumin and immunoglobulin, has been demonstrated to enable improved detection of low-abundant proteins. However, conventional affinity technologies are designed to remove one protein at a time. The Agilent Multiple Affinity Removal System is innovatively designed to remove multiple high-abundant proteins in a single step. As depicted in Figure 1, multiple targeted high-abundant proteins can be affinity captured by immobilized antibodies when passed through an Agilent Multiple Affinity Removal Column. The targeted proteins are albumin, IgG, antitrypsin,

IgA, transferrin, and haptoglobin representing 85%–90% of total human serum protein mass. After depletion of the six highly abundant proteins, lower-abundant proteins are recovered from the flow-through fraction and studied further, free of interference, enabling improved resolution on 1DGE, 2DGE and LC/MS.

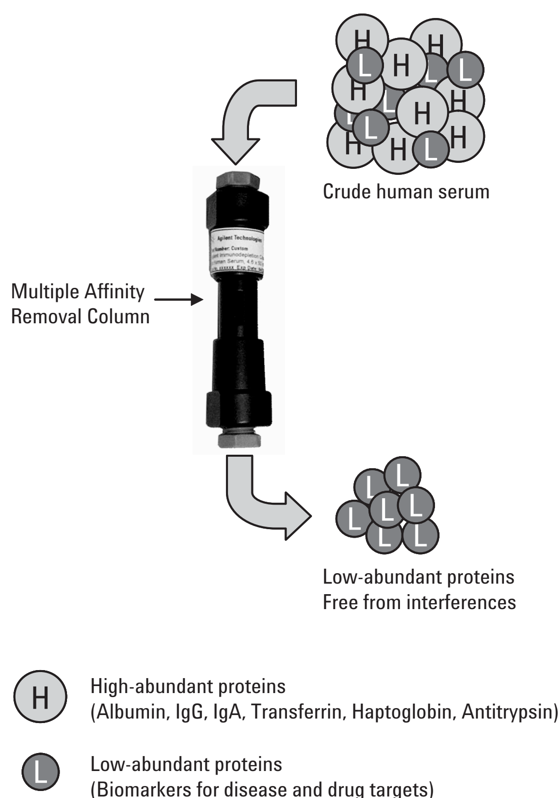

Figure 1. Principle of Multiple Affinity Removal Column.

Experimental

A sample of human sera was purchased from Sigma (St. Louis, MO). Agilent Multiple Affinity Removal System was developed, in house [1,2], at Agilent Technologies (Wilmington, DE). The system consists of an affinity column in 4.6 × 50 mm and 4.6 × 100 mm dimensions, Buffer A for sample loading, and Buffer B for eluting bound proteins. 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.

HPLC Protocol for Immunoaffinity Chromatography

Sample processing and fractionation were performed according to the protocol provided with the Agilent Multiple Affinity Removal System. Briefly, crude human serum was diluted five times with Buffer A containing protease inhibitors (COMPLETE, Roche). To remove particulates, the diluted serum sample was spun through a 0.22 µm spin tube at 16,000 × g at room temperature for 1–2 minutes. Automated sample injection onto a 4.6 × 50 mm affinity column was set for 75 µL in Buffer A (0% B) at a flow rate of 0.25 mL/min for 9 minutes. The bound fraction was eluted with Buffer B (100% B) at a flow rate of 1.0 mL/min for 3.5 minutes. Then, the column was re-generated by equilibrating with Buffer A (0% B) for 7.5 minutes with a total run cycle of 20 minutes. Flow-through and bound fractions were collected at 1.5–4.5 minutes and 10–11.5 minutes, respectively. Consecutive injections of a diluted human sera sample were set for 200 runs under standard conditions with fractions collected automatically into 1.5 mL tubes.

Protein Analysis by SDS-PAGE

To visualize the protein pattern of human sera before and after depletion, the collected fractions were spun through a 5 kDa molecular weight cutoff (MWCO) concentrator with buffer exchanged to 10 mM Tris-HCl pH 7.4 at 7500 × g for 20 minutes. Equal amounts of protein mass from each fraction were resolved in Novex 4%–20% Tris-Glycine gels (Invitrogen, Carlsbad, CA). Proteins were visualized by Coomassie blue staining. Protein content was estimated using a Pierce BCA protein assay kit.

Results and Discussion

A representative chromatogram from a 4.6 × 50 mm affinity column for removal of six high-abundant proteins from human serum is shown in Figure 2. The flow rate for sample loading at 0.25 mL/min is used for highly specific antibody-antigen recognition and binding. The buffer constituents allow efficient separation of the targeted high-abundant proteins from other proteins in human serum. Unlike conventional affinity columns that bind to one protein only, Agilent Multiple Affinity Removal Columns are packed with immobilized antibodies against six targeted proteins in approximately the same ratio as that found in serum. All six proteins are removed with high specificity when passed through the column, which saves a significant amount of time as compared to procedures for removal of each protein individually.

The flow rate for eluting bound proteins with Buffer B at 1.0 mL/min is four times as high as that

for sample loading. This is to ensure complete stripping of bound proteins from the column. As shown in Figure 2, high-abundant proteins appear as a high elution peak (absorbance at 280 nm) in a relatively short period of time. Then, the column is regenerated by re-equilibrating with Buffer A for 7.5 minutes. Each run cycle takes 20 minutes of total run time, resulting in efficient removal of all six high-abundant proteins from the low-abundant serum proteins. The small peak at 15 minutes has no proteins and might reflect changes in buffers from B to A.

It is well known that the chromatographic performance of protein separation can be influenced by conditions such pH, ionic strength, or the constituents of the mobile phases. To avoid run-to-run variability and to provide a reliable technology for proteomics sample preparation, the two buffers in the system are optimized for promoting maximum binding capacity (sample loading and selectivity), column lifetime (elution and regeneration) and reproducible sample fractionations. As shown in Figure 2, using the standard LC conditions, chromatographic consistency was observed over 200 consecutive injections (overlay of chromatograms) of a human serum sample on the same column.

As shown in Figure 3a, the high-abundant proteins in crude human serum (Figure 3a, lane 2) were efficiently removed from the flow-through fraction (Figure 3a, lane 3). Moreover, only background levels of the six proteins were detected in flow-through fractions using enzyme-linked immunosorbent assay (ELISA)[2]. Tryptic in-gel digestion of proteins from eluted fraction (Figure 3a, lane 4), combined with LC/MS/MS determined that Apolipoprotein 1, complement C3 and C4 were the only nontarget proteins detected in the bound fraction [3]. Nontargeted proteins detected in the bound fraction represent only a small fraction of the flow-through quantities. Figure 3b shows a consistent protein pattern on 1DGE of flow-through fractions of human serum from run number 20 to 200, demonstrating excellent reproducibility of sample fractionation by the affinity column.

Conclusion

The Agilent Multiple Affinity Removal System is efficient and reliable for simultaneously removing six targeted high-abundant proteins from human serum, allowing the enrichment and visibility of low-abundant proteins of interest. Current separation technologies for proteomics such as 1DGE, 2DGE and MD-HPLC/MS will benefit from the enabled increase in mass loading (~10 times) of low-abundant proteins. The system captures and removes the targeted high-abundant proteins with high specificity, ease of use, short operation time, and chromatographic reproducibility. The system can be also applied to other human body fluids such as plasma and CSF. Finally, use of an automated LC station for sample handling ensures

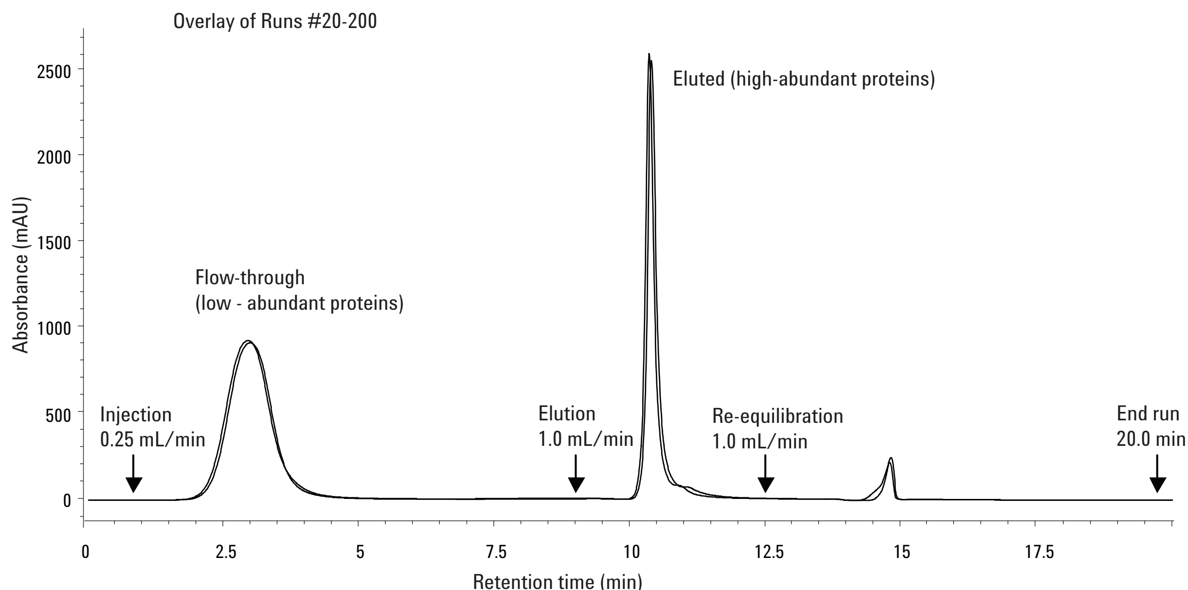

Figure 2. Chromatogram of the affinity removal of high-abundant proteins from human serum. Consecutive injections of 75 µL of 5× diluted human serum were made on a 4.6 × 50 mm Multiple Affinity Removal Column at a flow rate of 0.25 mL/min in Buffer A. Depleted protein fractions (flow-through) were collected 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 minutes. Identical chromatograms were obtained when overlaying runs number 20 and 200 on an Agilent 1100 LC System. This result indicates high chromatographic reproducibility during 200 runs.

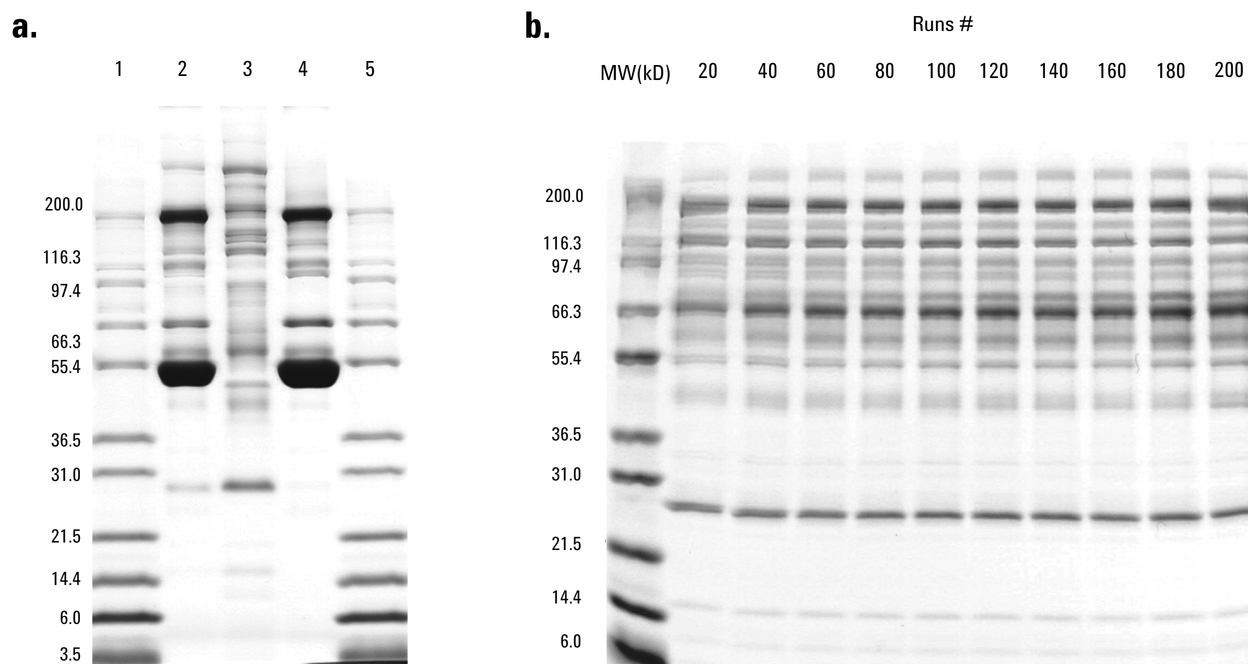

Figure 3. Enhanced detection of low-abundant human serum proteins after the six targeted proteins were removed by a Multiple Affinity Removal Column. Protein concentration was determined using a Pierce BCA protein assay kit with BSA as a standard. a. Equal amounts of protein (10 µg) from each fraction were separated by 4%–20% SDS PAGE under nonreducing conditions and visualized by Coomassie Blue staining. lane 2: unprocessed human serum. lane 3: flow-through low-abundant protein fraction, and lane 4: bound high-abundant protein fraction. Lanes 1 and 5 are molecular weight markers (Mark12 Invitrogen). b. Efficient removal of high-abundant proteins with high reproducibility for at least 200 injections. Aliquots of flow-through fractions from every 20th up to 200th injections of 1.4 mg total human serum protein were resolved by 4%–20% SDS PAGE. Excellent reproducibility of low-abundant protein constituents was indicated by a consistent gel pattern of the enriched proteins.

run-to-run consistency and increases sample processing throughput. The Agilent Multiple Affinity Removal System provides a practical and reproducible technology for removal of high-abundant proteins from human plasma or serum samples for downstream processing and protein identification.

References

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