Agilent Multiple Affinity Removal Spin Cartridge for Human Serum

Application

Proteomics

Authors

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Abstract

The Agilent Multiple Affinity Removal System comprises a family of immunodepletion products based on antibody-antigen interactions and optimized buffers for sample loading, washing, eluting, and regenerating. This spin cartridge is specifically designed to remove six high-abundance proteins from human biological fluids such as serum, plasma, and cerebral spinal fluid (CSF). This technology enables removal of albumin, IgG, antitrypsin, IgA, transferrin, and haptoglobin in a single step. Specific removal of six high-abundant proteins depletes approximately 85%–90% of total protein mass from human serum. Results indicate equivalent performance (efficiency and specificity) as Agilent Multiple Affinity Removal LC columns, with a complete depletion and regeneration in approximately 10 minutes. The spin cartridge also offers the ability to process multiple samples at one time.

Introduction

Protein biomarker discovery relies upon the identification of proteins typically in low abundance when compared to overall protein levels in human serum or plasma. Human albumin represents about 54% of the total protein in human serum, while IgG, IgA, transferrin, haptoglobin, and α-1-antitrypsin represents about 30% of the total protein in human plasma. The relative abundance of these proteins makes the discovery and identification of novel biomarkers a challenge. The ability to remove one of these high-abundant constituents, though reducing the complexity, still leaves a large amount of unwanted protein in samples. The remaining high-abundant proteins can still interfere with LC/MS analysis, making identification of the low-abundant proteins difficult. Furthermore, the use of 2D-gels in the presence of such high-abundant proteins makes it difficult to see the proteins of interest.

The Agilent Multiple Affinity Removal Column provided LC users with a simple way to remove the six most abundant proteins in human serum from successive samples, thus reducing the complexity of the proteome. As a result, more proteins were able to be identified through LC/MS than previously with systems that removed only one protein and with much lower specificity. The LC column format, however, did not address the concerns of some biological laboratories where either there was no LC technology or no need for a complete and automated sample processing solution that the LC column provides. In addition, many laboratories have expressed interest in processing many samples at the same time, in a high-throughput fashion, which is not applicable to the LC column format.

The Agilent Multiple Affinity Removal Spin Cartridge provides a fast and reproducible method to remove the same six most abundant proteins in human serum. The cartridges have about half the capacity of the 4.6 × 50 mm Agilent Multiple

Affinity Removal LC Column for serum, typically about 7–10 µL versus 15–20 µL, respectively. The spin cartridge system requires a centrifuge for processing samples and a syringe for preparing the cartridge for successive runs. This protocol may be completed in approximately 10 minutes, while samples may be further processed or subjected to 1D- or 2D-gels. The Agilent Multiple Affinity Removal Spin Cartridge provides an accurate, fast and reproducible method to remove between 85%–90% of the most abundant proteins in human serum for laboratories that do not require automation or for laboratories performing multi-sample processing simultaneously.

Experimental

The Multiple Affinity Removal Spin Cartridge System for removing albumin, transferrin, IgG, IgA, haptoglobin, and antitrypsin from human serum is a product from Agilent Technologies, Inc. (Wilmington, DE). A 0.45-mL spin cartridge (Agilent p/n 5188-5230) with binding capacity for 500 µg or 7–10 µL of human serum was used for these tests.

Spin Cartridge Protocol

Sample processing and fractionation were performed according to the protocol provided with the Agilent Multiple Affinity Removal Spin Cartridge System (Figure 1). Briefly, crude human serum was diluted with Buffer A (p/n 5185-5987) 20–30-fold based on the value of serum capacity indicated on the certificate of analysis supplied with each spin tube. The sample was filtered through a 0.22-µm spin filter (p/n 5185-5990) at 16,000 × g at room temperature for 1–2 minutes to remove particulates. Prior to loading the sample, the spin cartridge is flushed with 2 mL of Buffer A using a syringe. The sample is loaded directly onto the Multiple Affinity Removal Spin Cartridge, spun at 100 × g for 1.5 minutes, and the flow-through fraction is collected in an a collection tube (p/n 5188-5251). An additional 400 µL of Buffer A is added to the spin cartridge and centrifuged at 100 × g for 2.5 minutes, collecting into the same tube as previous. The spin cartridge is then placed in a new collection tube to which another aliquot of 400-µL Buffer A is added to remove any remaining low-abundant proteins and centrifuged at

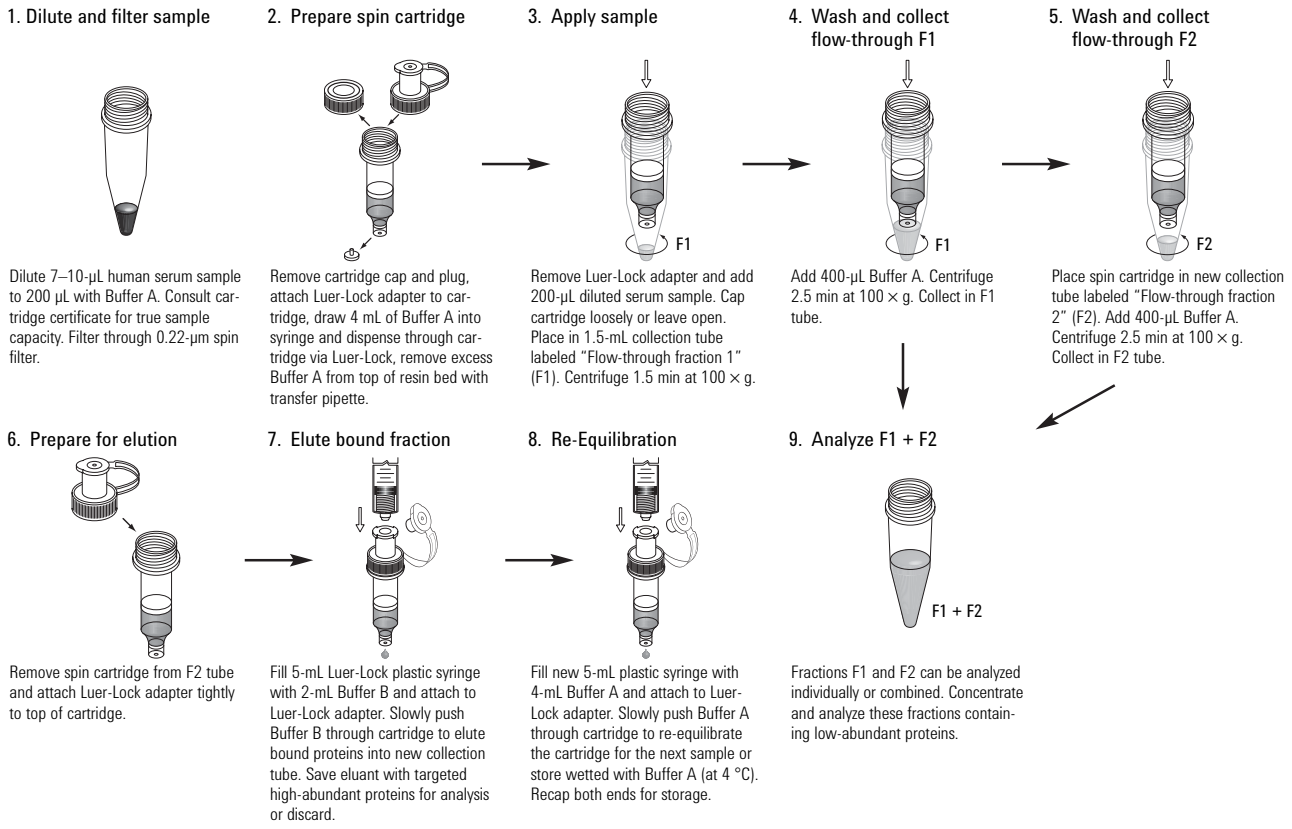

Figure 1. Protocol outline for use of the Agilent Multiple Affinity Removal Spin Cartridge for Human Serum/Plasma.

100 × g for 2.5 minutes. The resulting flow-through fractions may be analyzed individually or combined and concentrated for analysis. Using a syringe (p/n 5188-5250) and Luer-Lock adapter (p/n 5188-5249), the spin cartridge is then washed with 2-mL Buffer B (p/n 5185-5988) to elute the bound protein fraction, followed by re-equilibration with 4-mL Buffer A before the next run (or before storage at 4 °C). The analysis was performed 200 times on the same spin cartridge.

Protein Analysis by SDS-PAGE

Analysis of fractions by SDS-PAGE was performed as previously described (Barrett et al, publication 5989-1347EN) [1]. Briefly, flow-through fractions are analyzed directly without manipulation. Bound fractions may be analyzed by performing a buffer-exchange and concentration using spin concentrators with a 5 kDa molecular weight cutoff (MWCO) according to supplied protocols (Agilent p/n 5185-5991). The buffer-exchange is performed three times. Protein concentrations for crude serum, flow-through fractions, and buffer-exchanged bound-fractions are determined using a Pierce BCA protein assay kit.

Proteins were loaded onto Novex 4%–20% Tris-glycine gels (Invitrogen, Carlsbad, CA) under nonreducing conditions. Proteins were visualized by GelCode Blue Staining (Pierce, Rockford, IL).

Protein Analysis by 2DGE

Flow-through fractions from five injections were pooled into a spin concentrator with 5 kDa MWCO. The samples were spun at 7,500 × g at 10 °C. Buffer exchange was performed three times with 4 mL of buffer containing 7 M urea, 2 M thiourea, 2% CHAPS and 100-mM DTT. Concentrated protein fractions containing approximately 200 µg of proteins were diluted to 350 mL in IEF rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 100-mM DTT, 0.2% Biolytes, pH 3–10, 0.001% bromophenol blue) and applied to IPG strips for overnight rehydration. IPG strips were focused for 40,000 Vh at 20 °C using a BioRad Protean IEF System (BioRad, Hercules, CA). The second dimension (SDS-PAGE) was carried out on a BioRad Criterion System. The gels were stained with GelCode Blue (Pierce, Rockford, IL).

1-Dimensional LC/MS Analysis of Fractions

Analysis of gel bands excised from gels was performed as previously described (Barrett et al, publication 5989-1347EN) using an Agilent LC/MSD SL Ion Trap. Briefly, the digested samples were analyzed by liquid chromatography/tandem mass spectroscopy (LC/MS/MS) with scan range of 400–2200 *m/z*, AutoMS settings of Prefer Double Charged Ions, active exclusion on, SmartFrag on, Peptide Mode on, and averages set to 4. LC separations were performed on a Zorbax 300SB-C18, 3.5 µm, 75-µm × 150-mm column. Data analysis for all LC/MS/MS experiments was performed with Agilent Spectrum Mill software.

Results and Discussion

The high-abundant protein depletion results with the Agilent Multiple Affinity Removal Spin Cartridge for human serum/plasma are similar to those obtained with the LC-column format. The major proteins are removed providing access to the next level of proteins as shown in Figure 2. Removal of the six high-abundant proteins unmasks proteins that were either too low in concentration or were co-migrating with the high-abundant proteins whether on gel electrophoresis or LC/MS.

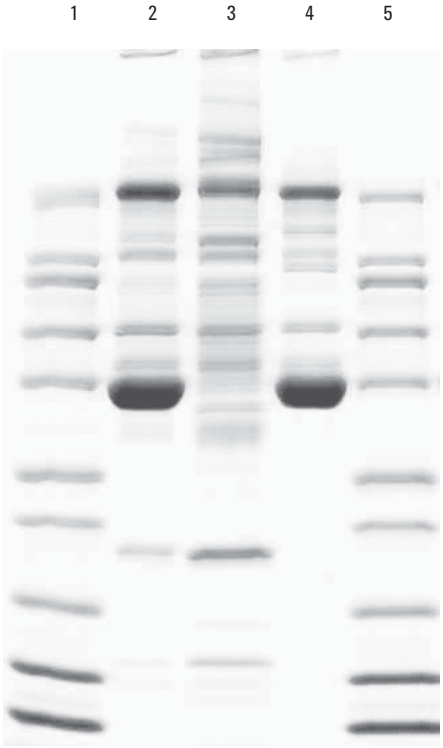

Figure 2. Enhanced detection of low-abundant human serum proteins after the six targeted proteins were removed by a Multiple Affinity Removal Spin Cartridge. Equal amounts of protein from each fraction were separated by 4%–20% SDS-PAGE under nonreducing conditions and visualized by GelCode staining. Lane 1, 5 - molecular weight standards, - Mark12; Lane 2 - crude serum, 9 µg; Lane 3 - flow-through fraction, 9 µg; Lane 4 - bound fraction, 9 µg.

Specificity of the spin cartridges for proteins removed from human serum was tested by LC/MS/MS. The bound fraction of proteins were eluted and run on a 1D-gel, bands were excised, reduced, alkylated, and digested with trypsin. The proteins were analyzed on an Agilent LC/MS Ion Trap and proteins analyzed via Spectrum Mill software. The results in Table 1 show good specificity for the six proteins targeted by the spin cartridge, while also removing IgE, IgA, and IgM heavy chains.

Table 1. Identified from Corresponding Gel Bands in the Bound-Fraction from the Multiple Affinity Removal Spin Cartridge

Protein
Serum albumin
Transferrin
Alpha-1-antitrypsin
Immunoglobulin G heavy chain
Immunoglobulin light chain (kappa and lambda)
Immunoglobulin M heavy chain
Immunoglobulin A heavy chain
Immunoglobulin E heavy chain
Haptoglobin

The Multiple Affinity Removal Spin Cartridge has the same robust performance as the LC-column format. As shown in Figure 3, the spin cartridge is able to carry out over 200 runs with excellent reproducibility. The 2D-gel results for crude serum and serum that was depleted of the targeted high-abundant proteins are shown in Figure 4. Clearly, removal of the targeted high-abundant proteins makes proteins spots that were masked by them more easily observed and accessed for proteomics studies.

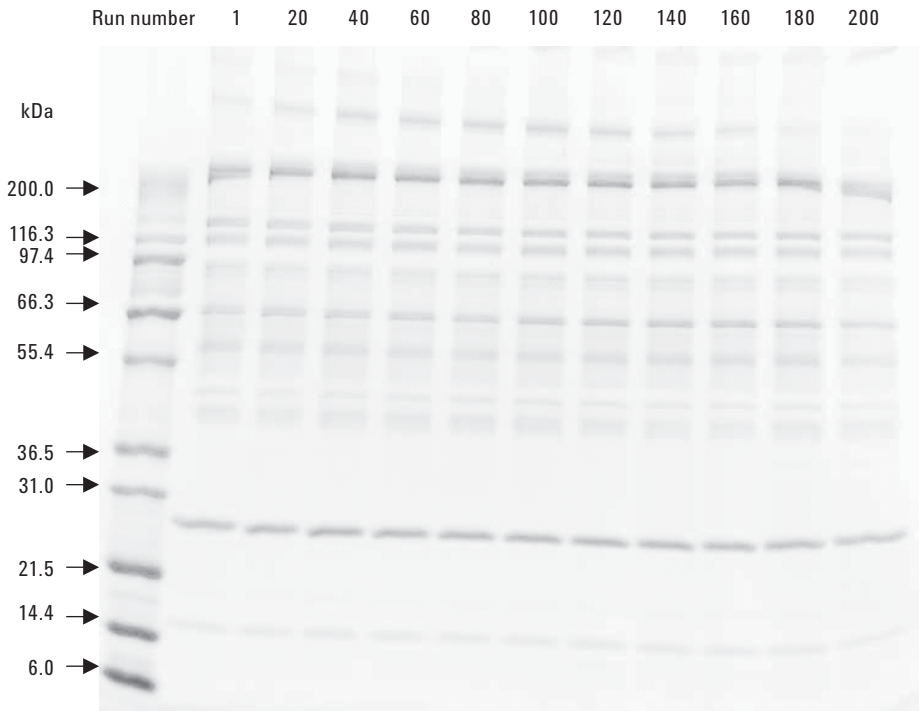

Figure 3. Efficient removal of high-abundant proteins with high reproducibility for at least 200 applications. Aliquots of flow-through fractions from every 20th up to 200th injections were resolved by 4%–20% SDS-PAGE. Excellent reproducibility of low-abundant proteins is indicated by a consistent gel pattern of the immunodepleted serum.

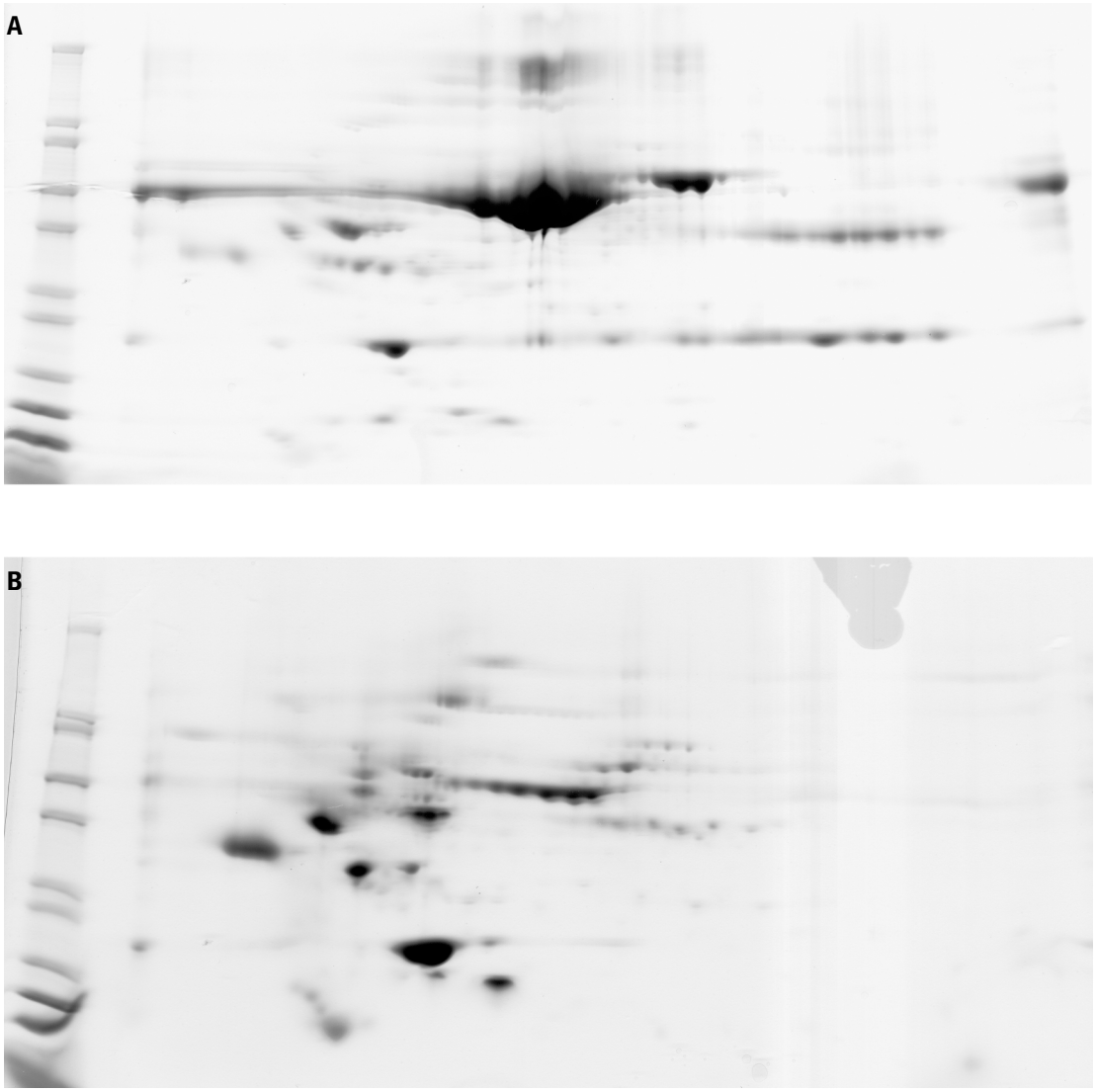

Figure 4. **Human serum protein patterns in GelCode stained 2D-gels. First dimension IPG strips (pH 3–10 nonlinear, 11 cm) for 40,000 Vh. Second dimension was a vertical gradient slab gel 4%–20%. Sample loading 200 µg of serum proteins. A. Crude serum, human; B. Immunodepleted serum from the Agilent Multiple Affinity Removal Spin Cartridge.**

Conclusions

The ability to simultaneously prepare samples for immunodepletion is a bottleneck in analysis of multiple proteomic samples. In addition, some laboratories that do not have access to LC systems have a need to deplete high-abundant proteins from serum and require alternative formats. The Agilent Multiple Affinity Removal Spin Cartridge provides a fast, reliable and reproducible method to deplete the six most abundant proteins in human serum/plasma including albumin, IgG, IgA, transferrin, antitrypsin, and haptoglobin. The spin cartridge provides access to the next level of proteins in the proteome. Further, the ability to process six samples using conventional microcentrifuge equipment in 10 minutes provides a way for researchers to simultaneously prepare many samples for additional downstream applications. This technology will further allow researchers to investigate the proteome through differential comparison from multiple samples without the need for an LC system.

Reference

1. Barrett, et al, “Removal of Multiple High-Abundant Proteins from Mouse Plasma Using the Agilent Multiple Affinity Removal System for Mouse,” Agilent Technologies, publication 5989-1347EN.
www.agilent.com/chem

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem/bioreagents.

Email technical inquiries to affinity_removal@agilent.com

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