Enhanced High Capacity Affinity Depletion of the Top Six Proteins from Human Serum

Application

Proteomics

Author

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Abstract

Human serum is easily accessible and the most complex sample of the human proteome. It is frequently used for the discovery of new disease markers, drug targets or studying protein expression patterns in human biology. Challenges arise in the proteomic analysis of serum due to the extreme concentration range (10–12 orders of magnitude) and structural complexity of its constituents, as well as the dynamic range limitations of current analytical techniques. The presence of high-abundant proteins such as HSA, IgG, haptoglobin, transferrin, IgA and antitrypsin interfere with the detection and analysis of low-abundant proteins of interest. The depletion of these six proteins (about 85% of the total protein mass in human serum) proves to be advantageous in the detection and identification of low-abundant proteins.

Introduction

A previous report details the development of a Multiple Affinity Removal Column, which permits highly specific and reproducible depletion of the top six high-abundant proteins in human serum. Serum column capacities, defined as the amount of undiluted serum which can be depleted of targeted high-abundant proteins with 99% efficiency, for at least 200 injections, were 20 µL for a 4.6 × 50 mm and 40 µL for a 4.6 × 100-mm column.

Now a significant improvement in the current affinity column technology allows at least twice the loading capacity of human serum. Two-dimensional LC/MS/MS analysis was used to determine the specificity of depletion, revealing that the higher capacity column is superior to available affinity depletion technologies. This column also provides robust and reproducible performance for at least 300 injections of human serum.

The high-capacity depletion column enables an expanded dynamic range for the detection of low-abundant proteins and sets the stage for 'digging deeper' into the human proteome in the search for novel biomarkers.

Experimental

Affinity Column

The newly developed column is a revision and improvement on the Agilent Multiple Affinity Removal approach described and evaluated previously [1, 2, 3]. This material is made with a novel attachment process for affinity binders, including, and improved from, the affinity-selected polyclonal IgG-based antibodies that were previously employed. The column requires a two-buffer system for operation. Buffers A and B are optimized to minimize co-adsorption of nontargeted proteins and to ensure reproducibility of column performance and long column lifetime. Buffer A - a salt-containing neutral buffer, pH-7.4, used for loading, washing and re-equilibrating the column and Buffer B - a low pH urea buffer used for eluting the bound high-abundant proteins from the column. See Table 1 for gradient conditions (UV detection at 280 nm).

Table 1. HPLC Protocol for 4.6 mm × 100-mm Multiple Affinity Removal Column

LC Timetable				
	Time	%B	Flow rate	Max. pressure
1	0.00	0.00	0.500	120
2	10.00	0.00	0.500	120
3	10.01	100.00	1.000	120
4	17.00	100.00	1.000	120
5	17.01	0.00	1.000	120
6	28.00	0.00	1.000	120

Sample preparation

Before injection onto a High Capacity Multiple Affinity Removal Column, human serum (Sigma - S 7023) was diluted five times with Buffer A. The sample was transferred to a 0.22-µm spin filter and centrifuged for 1 min. at 16,000 × g to remove particulates. Diluted serum was kept at 4 °C.

ELISA analysis of the flow-through fraction

Standard sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine the completeness of removal of targeted proteins from human serum. Briefly, assay plates were coated with 100 µL of flow-through fraction proteins diluted 1:10 in Buffer A. After an overnight incubation at 4 °C, plates were washed with phosphate buffered saline (PBS) and the nonspecific binding sites were blocked with 200 µL of blocker solution (Bio-Rad) for 2 hours.

After washing plates with PBS, 100 µL of diluted affinity-purified rabbit anti-human antigen antibodies were added in blocker solution. Plates were incubated for 2 hours, washed, and a secondary antibody HRP-conjugated goat anti-rabbit IgG (Sigma) was added for 1 hour. After washing with PBS, liquid substrate 3, 3', 5, 5', tetramethylbenzidine (TMB) was added and the absorbance was measured at 655 nm.

SDS-PAGE

Flow-through fraction proteins were loaded onto an SDS-PAGE without manipulations. Bound fractions were buffer-exchanged into 20 mM Tris-HCl in 4-mL spin concentrators with 5 kDa MWCO (Agilent Technologies 5185-5991) before loading onto gels. Protein concentrations were analyzed using a BCA protein assay kit (Pierce). Samples were stored at –70 °C until analysis. SDS-PAGE analysis was carried out using Invitrogen Tris-Glycine pre-cast gels (4%–20% acrylamide, 10 wells, 1 mm). Proteins were visualized by Coomassie Blue staining with GelCode Blue (Pierce).

LC/MS/MS Analysis

To analyze the specificity of immunodepletion, the bound fraction was resolved by SDS-PAGE. Coomassie Blue-stained protein bands were cut and processed with "In-gel trypsin digestion kit" (Agilent Technologies 5188-2749). Peptides were analyzed by LC/MS/MS on a SL TRAP (Agilent Technologies). Results were processed by Spectrum Mill software (Agilent Technologies).

For direct analysis of the bound fraction, the sample was buffer-exchanged into an ammonium bicarbonate solution, reduced, alkylated and digested with trypsin under denaturing conditions. Peptides were analyzed by 2D nanoLC/MS/MS XCT TRAP (Agilent Technologies) using a ZORBAX Bio-SCX Series II column for the first dimension, ZORBAX 300SB-C18, 5 µm, 5 mm × 300 µm as an enrichment column, and a reversed-phase (RP) column ZORBAX 300SB-C18, 3.5 µm, 150 mm × 75 µm for the second dimension (Agilent Technologies).

- Salt Steps: sample (0), 10, 20, 50, 100, 200, 500, 1000-mM NaCl.
- RP Mobile Phase: H_2O with 0.1% formic acid (FA) and acetonitrile (ACN) with 0.1% FA - 300 nL/min
- Enrichment Mobile Phase: H_2O with 0.1% trifluoroacetic acid (TFA) and 3% ACN - 15 µL/min
- RP Gradient: 3% B at 0 min., 3% B at 5 min., 15% B at 11 min., 45% B at 100 min., 85% B at 105 min., 85% B at 108 min., 3% B at 110 min.

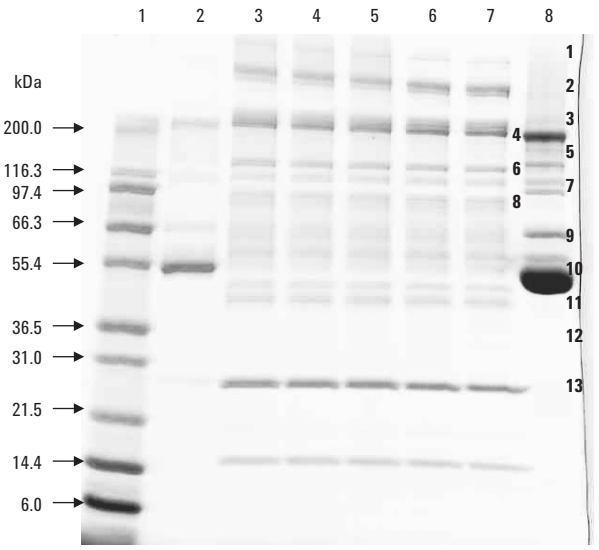
Results and Discussion

Specificity

The Agilent High Capacity Multiple Affinity Removal Column uses the same buffers and protocols as the Multiple Affinity Removal Column to deplete the six major proteins in serum including albumin, IgG, transferrin, haptoglobin, IgA, and α1- antitrypsin. It is specifically designed to remove these six high-abundant proteins from human biological fluids such as serum, plasma, cerebral spinal fluid (CSF), etc. These targeted high-abundant proteins are simultaneously removed when crude biological samples are passed through the column. Depleting these high-abundant proteins enables you to load more low-abundant proteins onto gels or for LC/MS/MS analysis.

To determine column capacity, a series of injections were made with increasing amount of human serum. The flow-through fractions were loaded directly onto gels and proteins were visualized by Coomassie Blue staining. The resulting patterns were consistent (Figure 1 - lanes 3–7) showing no breakthrough of the targeted high-abundant proteins. Because no breakthrough of the targeted proteins was visualized in the flow-through fraction (lane 7 [50-µL load]), the capacity of the High Capacity Multiple Removal Column is shown to be at least twice that of the current Multiple Affinity Removal Column.

The bound fraction from the High Capacity Multiple Affinity Removal System was buffer-exchanged and proteins were analyzed using 1DGE and visualized by Coomassie Blue staining (Figure 1 - lane 8) in order to determine if any additional proteins bind nonspecifically to either the column or targeted proteins. In-gel tryptic digests of proteins from the bound fraction (Figure 1 - lane 8 [bands 1–13]) were analyzed by LC/MS/MS. In addition, the bound fraction was directly digested (in-solution) and analyzed by 2D LC/MS/MS. The results are listed in Tables 2 and 3. IgM was the only nontargeted protein detected in the bound fraction and represents only a small amount relative to its mass in the flow-through fraction.



4%–20% SDS-PAGE

Figure 1. 1DGE of bound and flow-through fractions from a 4.6 × 50 mm High Capacity Multiple Affinity Depletion Column. Lane 1 - Mark12 Standards, Lane 2 - Serum, 3 µg, Lane 3 - Flow-through, 20-µL column load, Lane 4 - Flow-through, 30-µL column load, Lane 5 - Flow-through, 40-µL column load, Lane 6 - Flow-through, 45-µL column load, Lane 7 - Flow-through, 50-µL column load, Lane 8 - Bound fraction, 9 µg. Protein bands marked in bold numbers (bound fraction) were cut and processed through in-gel trypsin digestion procedure, peptides were extracted and analyzed by LC/MS/MS.

Table 2. Proteins Identified by LC/MS/MS Analysis of Gel Bands (Figure 1, Lane 8) of Bound Fraction

Band number	Proteins identified
1	Keratin
2	Keratin
3	HSA, IgG
4	IgG, Keratin
5	HSA,IgG, IgA
6	HSA, Alpha 1-antitrypsin, IgG
7	Haptoglobin, HSA
8	HSA, Haptoglobin, IgA
9	Transferrin, HSA
10	HSA, Alpha 1-antitrypsin
11	HSA
12	HSA, IgG
13	Not identified

Table 3. Direct Two-Dimensional LC/MS/MS of Bound Fraction

Band number	Proteins identified
1	HSA
2	Transferrin
3	Alpha 1-antitrypsin
4	IgG
5	Haptoglobin
6	IgM
7	IgA
8	Trypsin

Completeness of Immunodepletion

The ELISA data in Table 4 indicates greater than 99% depletion of the six targeted proteins on both the 4.6 × 50-mm and the 4.6 × 100-mm column. This reliable depletion is consistent from run to run and from column to column, allowing parallel studies to be performed with confidence.

Table 4. ELISA Data of Bound Fractions of Hu-6HC Columns. Fifty Microliters of Human Serum Were Injected on the 4.6 × 50-mm Column and 100 µL of Human Serum Were Injected on the 4.6 × 100-mm Column

Targeted protein	Min. achieved depletion 4.6 × 50-mm column (%)	Min. achieved depletion 4.6 × 100-mm column (%)
HSA	99.6	99.8
Transferrin	99.6	99.8
IgG	99.6	99.8
Haptoglobin	99.6	99.8
IgA	99.6	99.8
Alpha1-antitrypsin	99.6	99.8

Reproducibility and Robustness

Reproducibility of the High Capacity Multiple Affinity Removal System was tested by the analysis of 300 injections on a single column (Figure 2). The two buffers provided with the system are optimized for promoting maximum binding capacity (sample loading and selectivity), column lifetime (elution and regeneration), and reproducible sample fractionation. Figure 3 shows a consistent protein pattern on 1DGE of flow-through fractions of serum from run number 1 to 300, indicating excellent reproducibility and robustness.

Comparison

Table 5 shows the enhanced capacity values of the new High Capacity Multiple Affinity Columns, which can deplete at least twice the amount of serum per injection (compared to the previous Multiple Affinity Removal Columns) while retaining the same high specificity. Robustness is also enhanced to more than 200 runs, with data shown in Figures 2 and 3 for 300 runs.

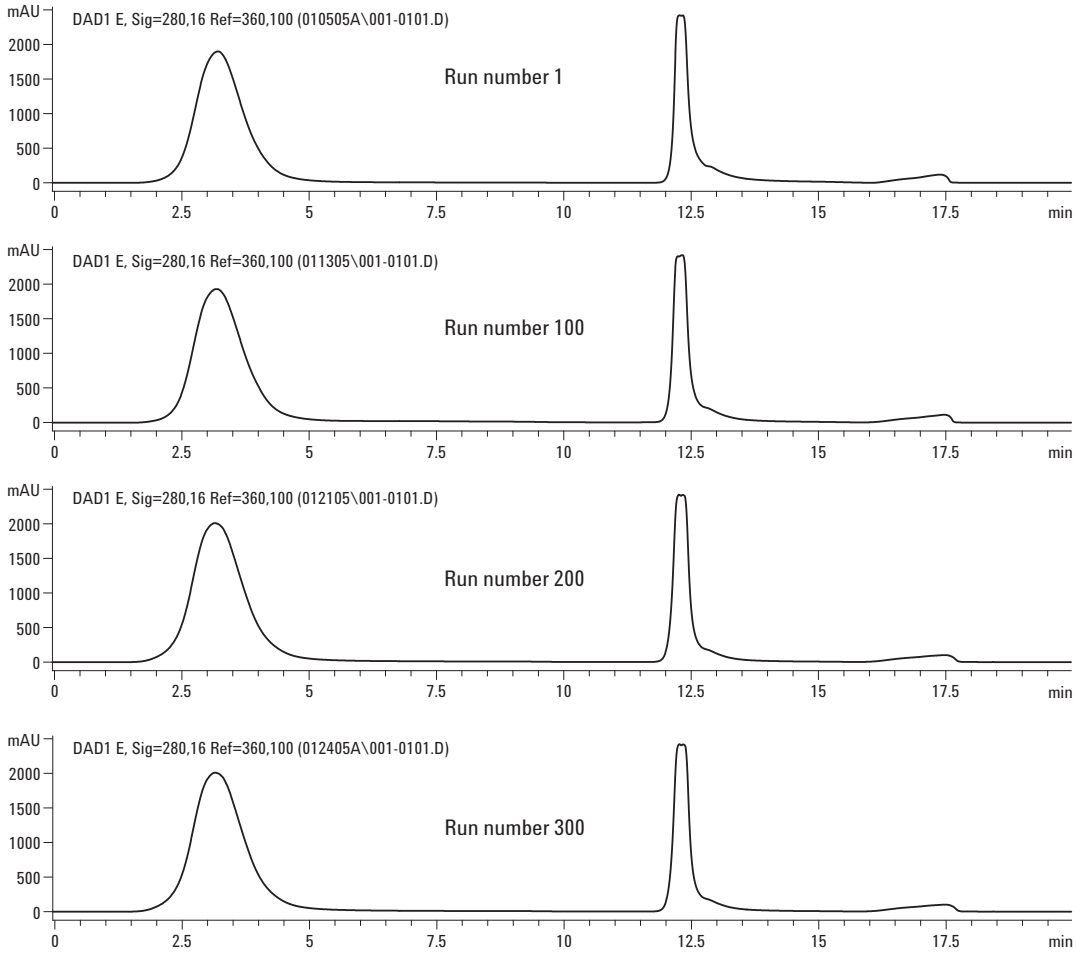
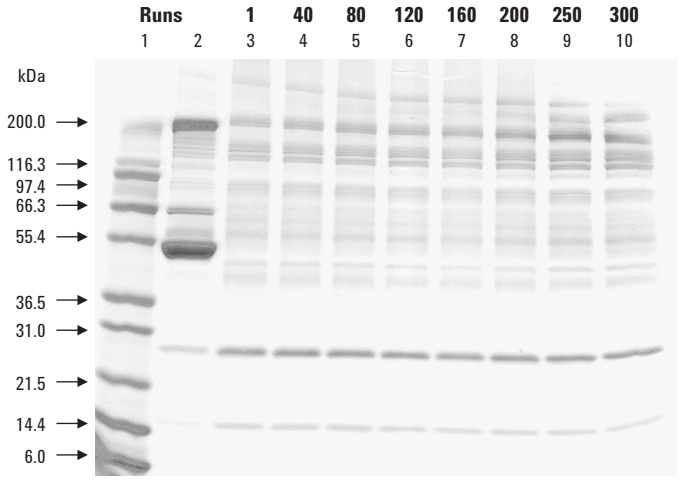


Figure 2. LC Chromatograms during 300 runs of the affinity removal of high-abundant proteins from human serum. Crude serum was diluted four-fold with Buffer A and filtered prior to loading onto the column. The sample was loaded at a flow rate of 0.5 mL/min with Buffer A (100%) and allowed to run for 9 minutes. The bound fractions were eluted with Buffer B (100%) for 4 minutes followed by re-equilibration for 7 minutes with Buffer A (100%).



4%–20% SDS-PAGE

Lane 1 - Mark12 Standards
Lane 2 - Serum, 8 µg
Lanes 3–10, Flow-through fractions, runs 1–300

SDS-PAGE results indicate no breakthrough of the targeted proteins in flow-through fractions up to 300 runs!

Figure 3. Analysis of column performance during 300 runs. Enhanced detection of low-abundant serum proteins after the six targeted proteins were removed by a High Capacity Multiple Affinity Removal Column. Excellent reproducibility of low-abundant protein constituents was indicated by a consistent gel pattern of the enriched proteins.

Table 5. Column Comparison Between Multiple Affinity Removal Columns and High Capacity Multiple Affinity Removal Columns

Column properties	Multiple Affinity Removal Column*	High Capacity Multiple Affinity Removal Column
Capacity µL of serum loaded	4.6 × 50 mm - 15–20 µL	4.6 × 50 mm - 30–40 µL
	4.6 × 100 mm - 30–40 µL	4.6 × 100 mm - 80–100 µL
Specificity	High	High
Completeness of depletion	>99%	>99%
Robustness	Reproducible depletion for at least 200 runs	Reproducible depletion for more than 200 runs (300 runs tested)

*Data obtained from the Multiple Affinity Removal Column manual

Conclusion

While the enhanced High Capacity Multiple Affinity Removal Columns exhibit the same specificity as our previous columns, they provide at least twice the capacity and greater robustness for the depletion of six high-abundant proteins. The new columns are extremely robust and perform reproducibly for 300 runs. Analysis of column specificity by LC/MS/MS revealed minimal untargeted proteins in column bound fraction. The LC column format is easy to use and lends itself to automation for reproducible and consistent depletion.

Increased capacity for the removal of interfering high-abundance proteins enables use of larger starting samples for subsequent fractionation and analysis, expanding the dynamic range of proteomics analysis, thus assisting scientists in biomarker discovery and validation.

References

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