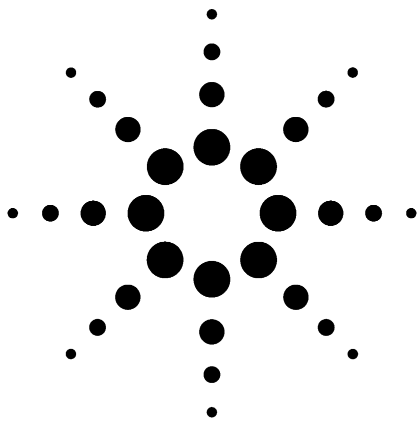

Removal of Multiple High-Abundant Proteins from Mouse Plasma Using the Agilent Multiple Affinity Removal System for Mouse

Application

Proteomics

Authors

William Barrett, Nina Zolotarjova, Haiying Chen, Peter Mrozinski, Cory Szafranski, Jerome Bailey and Barry Boyes
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

The study of biological samples in disease models or in drug therapy requires the ability to identify proteins, which are present at relatively low concentrations. This analysis is compromised by the presence of a few proteins that are present at relatively high concentrations, which interfere with the ability to detect low-abundant proteins. The Agilent Multiple Affinity Removal System for human serum provides a fast and reproducible method to remove six high-abundant proteins from human samples including serum, plasma, and cerebrospinal fluid (CSF). However, many researchers are using murine models in disease, toxicology, and drug testing to identify protein biomarkers. Once again, there are mixed results with available products in the depletion of low-abundant proteins in murine models. The Agilent Multiple Affinity Removal System for mouse serum addresses this concern by removing three high-abundant proteins including albumin, IgG, and transferrin from mouse serum simultaneously and reproducibly using an LC-column format. The column depletes >98%–99% of the three targeted proteins with robust performance over 200 runs, similar to the human protein removal column. In addition, the column effectively removes the same three targeted proteins in rat serum as well. The Agilent Multiple Affinity Removal System for mouse serum provides another tool that enables scientists to expand the dynamic range of proteomic analysis.

Introduction

There are major obstacles to the discovery of novel protein biomarkers in drug development and disease models. The ability to detect low-level proteins in mouse serum is hindered by the presence of albumin, IgG, and transferrin. Together, these three proteins can comprise 80% of the total protein mass. The depletion of these three abundant proteins facilitates access to the low-level proteins of interest. The Multiple Affinity Removal System for mouse is highly specific and compatible with subsequent fractionation of the depleted serum.

The immunoaffinity column is based upon polyclonal antibodies, which are purified via a stringent affinity purification method. The use of highly-purified mouse antigens for the purification of specific polyclonal antibodies generates antibodies that only cross-react with proteins from closely related species, such as rat. This specificity minimizes nonspecific interactions and eliminates the need for resins, such as those modified with Cibacron Blue, that are based on cofactor binding sites, which are more likely to interact with non-targeted proteins.

Experimental

The Multiple Affinity Removal System for mouse, which removes albumin, IgG, and transferrin from mouse serum, is a product from Agilent Technologies (Wilmington, DE). A 4.6 mm × 100-mm Multiple Affinity Removal column (part number 5188-5218) was used with a mobile phase reagent kit (Agilent part number 5185-5986). Injections of diluted serum, sample loading, washing, and column regeneration is done using Buffer A according to manufacturer protocols. The elution of bound proteins is done with Buffer B according to manufacturer protocols. Fractions were automatically collected using peak-based fraction collection into 1.5-mL plastic tubes (Sarstedt, Numbrecht, Germany) using an Agilent 1100 HPLC equipped with a thermostatted analytical scale fraction collector [1]. Depleted low-abundant proteins as well as eluted high-abundant bound proteins were collected and stored at –20 °C until analysis.

HPLC Protocol for Immunoaffinity Chromatography

The HPLC protocol for using the mouse immunoaffinity column is identical to the protocol for the human immunoaffinity column of 4.6 mm × 100-mm size. Briefly, mouse serum is diluted five-fold with Buffer A and samples are filtered through 0.22-µm filters (Agilent part number 5185-5990) by centrifugation at 16,000 × g at room temperature for 1 minute. Samples are then placed in a thermostatted autosampler with temperature set to 4 °C and sample injection was set for 250 µL per injection in 100% Buffer A at a flow rate of 0.5 mL/min for 10 minutes. The unbound low-abundant proteins were collected in a thermostatted fraction collector set at 4 °C using peak-based collection. The bound proteins were eluted in 100% Buffer B at a flow rate of 1.0 mL/min for 7 minutes. This step is followed by re-equilibration and regeneration in 100% Buffer A for 11 minutes (Table 1).

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

Solvent A:	Buffer A
Solvent B:	Buffer B
Pressure limits:	120 bar

LC Timetable

Event	Time, (min)	%B	Flow rate, (mL/min)	Max. pressure, (bar)
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

Preparation of Protein Fractions for 1-Dimensional Gel Electrophoresis (1DGE)

Flow-through fractions (low-abundant proteins) can be analyzed directly on 1D-gels. If desired, the bound fraction, containing high-abundant proteins, may be analyzed by 1D-gel as well. Bound fractions were buffer-exchanged and concentrated using 4-mL spin concentrators with a 5 kDa molecular weight cut-off (MWCO) according to supplied protocols (Agilent part number 5185-5991). Briefly, samples were centrifuged at 7,500 × g for 20–25 minutes at 8 °C. Buffer-exchange was performed by repeated centrifugation three times into appropriate buffer for downstream analysis. Protein concentration for crude serum, flow-through fractions and bound fractions (buffer-exchanged) were determined using a Pierce BCA protein assay kit.

Proteins were analyzed pre- and post-depletion on SDS-PAGE gels to determine reproducibility over 200 runs. Equal amounts of protein 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).

1D LC/MS Analysis

The mouse bound fraction was run on 1DGE as described above. The bands were excised and digested with trypsin using the Agilent Protein In-Gel Tryptic Digestion Kit (part number 5188-2749) as per the manufacturer's protocol. The digested samples were analyzed by liquid chromatography/tandem mass spectroscopy (LC/MS/MS) on an Agilent LC/MSD TRAP XCT. Briefly, the LC/MSD TRAP XCT conditions were a 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, 150-mm × 75-µm column, 0.5 µL injected, injector switched to bypass at 5 minutes, Solvent A: 0.1% formic acid in water, Solvent B: 0.1% formic acid in ACN, flow rate: 300 nL/min, column temperature at 40 °C with the gradient listed in Table 2. Data analysis for all LC/MS/MS experiments was performed with Agilent Spectrum Mill software.

Table 2. **LC Gradient for LC/MS Analysis of In-Gel Tryptic Digests of High-Abundant Proteins from 1DGE**

NanoLC pump gradient	
Time, min	% B
0	10
5	10
55	40
60	80
65	80
68	10

2D LC/MS Analysis

The mouse flow-through fraction was collected, desalted, buffer-exchanged into phosphate buffered saline (PBS), and subjected to an in-solution tryptic digest procedure. The sample was purified, concentrated, and then analyzed by 2D LC/MS/MS on an Agilent LC/MSD TRAP XCT. Briefly, the LC/MSD TRAP XCT was operated in nanoelectrospray with the Agilent orthogonal source (G1982B), conditions were a scan range of 400–2200 *m/z*, AutoMS settings of Prefer Double Charged Ions, Active Exclusion on, SmartFrag on, Peptide Mode on, averages set to 3, and MS/MS scan range of 100–1800 *m/z*.

The first dimension of LC separation was performed on an Agilent BioSCX Series II, 0.8 × 50-mm column. The second dimension LC separation used a ZORBAX 300SB-C18 3.5 µm, 150 mm × 75-µm with ZORBAX 300SB-C18, 5 µm, 0.5 × 35 mm as the enrichment column. The mobile phase for the first dimension was 0.1% formic acid in 3% ACN/water at 15 µL/min. For the second dimension, Solvent A: 0.1% formic acid in water, Solvent B: 0.1% formic acid in ACN, flow rate: 300 nL/min with the gradient listed in Table 2. Salt steps: 0, 10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 200 mM, 500 mM, 1 M, and 2.5 M of KCl, with 20 µL injected. Data analysis for all 2D LC/MS/MS experiments was performed with Agilent Spectrum Mill software.

Results and Discussion

The Agilent Multiple Affinity Removal Column for mouse serum uses the same buffers and protocols as the human Multiple Affinity Removal Column to deplete three major proteins in mouse serum including albumin, IgG, and transferrin. Since many scientists use murine models, the mouse Multiple Affinity Removal Column improves the dynamic range available for study. Based upon the successful platform of the Agilent Multiple Affinity Removal System for human serum, the mouse column has many of the same properties [2].

A representative chromatogram from a 4.6-mm × 100-mm Agilent Multiple Affinity Removal column for mouse serum (Agilent Part Number 5188-5218) is shown in Figure 1. The loading of the column is controlled with a lower flow rate (0.5 mL/min) to allow for optimal binding of high-abundant proteins while the low-abundant proteins flow through the column and are collected. The flow rate is increased for the elution of bound proteins and regeneration and equilibration of the column. The bound proteins may either be collected for analysis (for protein assays, the sample will need to be buffer-exchanged) or discarded. The flow-through fraction and the bound fraction of mouse serum were collected and concentrated for further analysis.

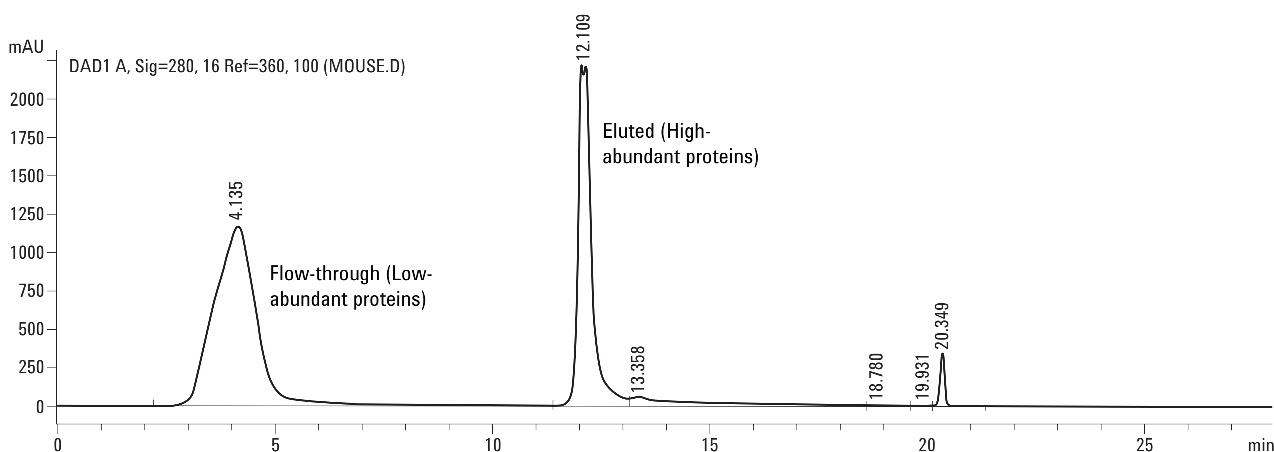

Figure 1. Chromatogram of the affinity removal of high-abundant proteins from mouse serum. Crude serum (50 µL) was diluted five-fold with Buffer A and filtered prior to loading onto the column. The sample was loaded at a flow rate of 0.5 mL/min with Buffer A (100%) and allowed to run for 10 minutes. The bound fractions were eluted with Buffer B (100%) for 7 minutes followed by re-equilibration for 11 minutes with Buffer A (100%).

As shown in Figure 2, the targeted proteins in crude mouse serum (Figure 2, lane 2) were efficiently removed from the flow-through fraction (Figure 2, lane 3). In addition, enzyme-linked immuno sorbent assay (ELISA) data indicates only background levels of the three proteins are detected in flow-through fractions (data not shown) indicating >98% depletion. Reproducibility of the Multiple Affinity Removal System for mouse serum was tested by the analysis of 200 injections on a single column. 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 2 shows a consistent protein pattern on 1DGE data of flow-through fractions of mouse serum from run number 1 to 200.

The eluted bound fractions from the Multiple Affinity Removal System for mouse were buffer-exchanged and proteins were analyzed using 1DGE and visualized by Coomassie Blue staining (Figure 3) 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 3) were analyzed by LC/MS and LC/MS/MS. The LC/MS/MS results are listed in Table 3. Apolipoprotein A-1, Complement C3, alpha-1-antitrypsin, alpha-2-macroglobulin and haptoglobin were the only non-targeted proteins detected in the bound fraction. The non-targeted proteins detected in the bound fraction represent only a small fraction of the flow-through quantities.

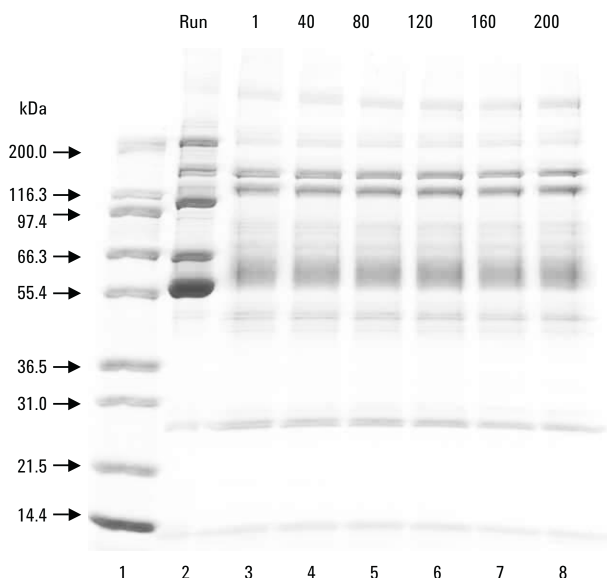

Figure 2. **Enhanced detection of low-abundant mouse serum proteins after the three targeted proteins were removed by a Multiple Affinity Removal Column. Aliquots of flow-through from every 20th up to 200th injection of mouse serum were resolved by 4%–20% SDS PAGE under nonreducing conditions and visualized by Coomassie Blue staining. Lane 1: molecular weight markers (Mark 12 Invitrogen). Lane 2: crude mouse serum. Lanes 3–8: flow-through low-abundant protein fractions. Excellent reproducibility of low-abundant protein constituents was indicated by a consistent gel pattern of the enriched proteins.**

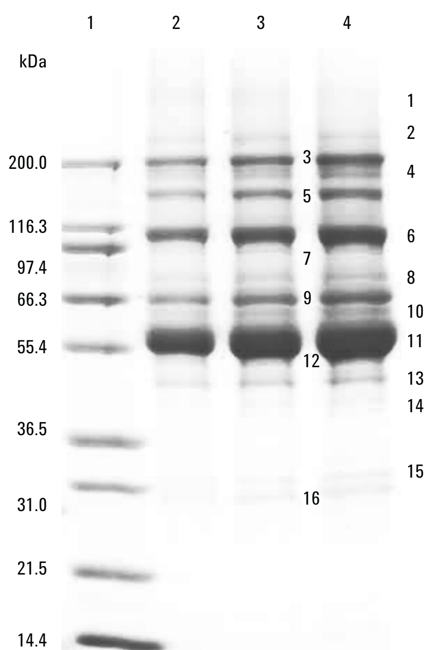

Figure 3. **Bound proteins eluted from Agilent Multiple Affinity Removal Column for mouse serum were resolved on SDS-PAGE, Coomassie Blue stained and labeled bands (1–16) were excised and subjected to in-gel tryptic digestion. Resultant peptides were analyzed by LC/MS and LC/MS/MS (See Table 3). Lane 1, molecular weight standards; Lane 2, bound high-abundant proteins 4 µg, Lane 3, bound high-abundant proteins, 8 µg; Lane 4, bound, high-abundant proteins, 12 µg.**

Table 3. Specificity of Mouse Multiple Affinity Removal Column as Determined by LC/MS/MS Analysis of Tryptic Digested Proteins from an Eluted Fraction of High-Abundant Proteins. Results were processed with Agilent Spectrum Mill software

	Protein name	Distinct peptides	Number of spectra
Band # 1	Serum albumin precursor	4	4
	Alpha-2-macroglobin precursor	2	2
Band # 2	Complement factor H precursor	6	7
	Murinoglobulin 1 precursor	2	2
Band # 3	Serum albumin precursor	7	7
	Immunoglobulin heavy chain	2	2
Band # 4	Serum albumin precursor	9	10
	Ig gamma-chain	2	2
Band # 5	Serum albumin precursor	13	13
	Alpha-1-antitrypsin 1–3 precursor	3	3
	Complement C3 precursor (HSE-MSF)	2	2
Band # 6	Serum albumin precursor	11	11
	Haptoglobin	1	1
Band # 7	Serum albumin	1	1
Band # 8	Serotransferrin precursor	14	15
	Serum albumin precursor	4	4
Band # 9	Transferrin	19	25
	Serum albumin precursor	8	8
	Serine proteinase inhibitor A3K precursor	6	6
Band # 10	Serotransferrin precursor	14	16
	Serum albumin precursor	9	12
	Alpha-1-antitrypsin	6	7
Band # 11	Serum albumin precursor	16	19
	Serotransferrin precursor	8	9
Band # 12	Serum albumin precursor	14	17
	Transferrin	6	6
Band # 13	Serum albumin precursor	12	13
Band # 14	Serum albumin precursor	6	6
Band # 15	Serum albumin precursor	2	2
Band # 16	Serum albumin	3	3
	Apolipoprotein A-1 precursor	2	2

The 2D/LC/MS/MS studies of the flow-through fraction identified 108 proteins as determined by Agilent Spectrum Mill Software. Identified within the list of proteins was kininogen, a biomarker for senescence in rats (R. Walter, et al., 1998), transcortin, which has been used in studies of septic shock and is typically present in the µg/mL range within serum (Beishuizen A, et al.), vitronectin which has correlated with interstitial lung disease (Pohl WR, et al.), and thrompospondin a serum protein involved with angiogenesis [3, 4, 5]. These results indicate that with depletion, previous proteins which might be masked by the high-abundant proteins may now be accessible for further studies.

Conclusion

The Agilent Multiple Affinity Removal Column for mouse serum is efficient and reliable for the simultaneous depletion of three high-abundant proteins from mouse serum. The column effectively depletes the three targeted proteins from rat serum with slight adjustments to capacity; these results will be published in a future application note. The column captures and removes the targeted proteins with high specificity. In addition, the format is easy to use and provides a consistent and reproducible platform for sample automation. Scientists will also be able to compare depleted serum profiles from mouse and human in the analysis of biomarkers. The depletion of high-abundant proteins results in an improved dynamic range on 2DGE or LC/MS, enabling the researcher to further simplify a complex system in the goal of discovering biomarkers.

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Email technical inquiries to: affinity_removal@agilent.com

William Barrett and Peter Mrozinski are Proteomics Application Scientists for Proteomics BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Nina Zolotarjova and Haiying Chen are R&D Scientists for Proteomics BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Cory Szafranski is Proteomics Product Manager for Proteomics BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Jerome Bailey is Marketing Manager for BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Barry Boyes is R&D Manager for BioReagents in the Integrated Biology Solutions Group with Agilent Technologies.

Correspondance: Dr. William Barrett

Email: bill_barrett@agilent.com

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