

Novel Immunoaffinity Depletion Technology With Increased Binding Capacity Removes Approximately 97% of High Abundance Human Plasma Proteins

Mark D. Schuchard, Christopher D. Melm, Angela S. Crawford, Holly A. Chapman,
Steven L. Cockrill, Kevin B. Ray, Richard J. Mehigh, William K. Kappel, and Graham B.I. Scott

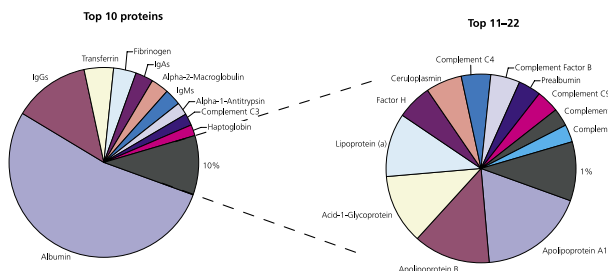
Abstract

We have developed a novel, high-binding capacity, antibody-based resin for the depletion of the high abundance proteins that represent approximately 97% of total protein in human plasma. This resin increases the number of proteins depleted to twenty (20) and is supplied in a convenient spin column format. Depletion of 97% of the total protein from human plasma allows for a 20–50-fold increase in the load of proteins of interest compared to whole plasma. This novel technology demonstrates the ability to deplete more proteins from more plasma, thus allowing the researcher a greater ability to visualize low copy number proteins in plasma samples and identify them by mass spectrometry.

Introduction

- The study of the human plasma proteome is an area of great interest, especially for the pharmaceutical potential of identifying disease biomarkers. Many proteins of pharmaceutical interest appear at low concentrations in the plasma and are therefore difficult to detect.
- Identification of potential biomarkers is especially difficult due to the presence of higher abundance proteins. Depletion of these abundance proteins allows for visualization of proteins that comigrate with, and are masked by, the high abundance proteins on 1DE or 2DE gels. Plasma proteins can then be loaded onto the gels or IPG strips at higher levels for improved visualization and detection of low copy number proteins.
- An affinity resin has been developed for removal of 20 high abundance proteins from 8 μ L of plasma. Depletion of these 20 high abundance proteins removes greater than 97% of the proteins in plasma, and permits loading of 20–50-fold more of each individual protein for improved visualization of lower copy number proteins.

Plasma Facts



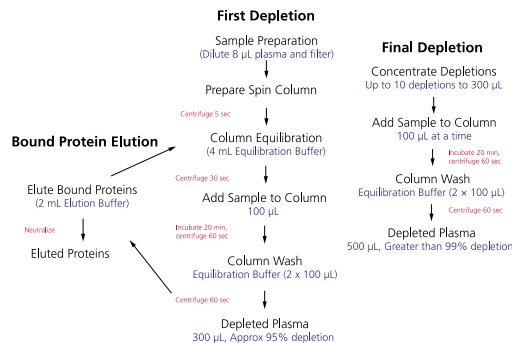
- The 10 most abundant proteins represent approximately 90% of the total protein mass in human plasma.
- The 22 most abundant proteins are said to represent approximately 99% of the total protein mass in human plasma.
- The PROT-20™ column removes the 20 high abundance plasma/serum proteins listed below. These 20 proteins represents approximately 97% of the total human plasma protein mass.

Albumin	Apolipoprotein A1
IgGs	Apolipoprotein A2
Transferrin	Apolipoprotein B
Fibrinogen	Acid-1-Glycoprotein
IgAs	Ceruloplasmin
Alpha-2-Macroglobulin	Complement C4
IgMs	Complement C1q
Alpha-1-Antitrypsin	IgDs
Complement C3	Prealbumin
Haptoglobin	Plasminogen

ProtePrep™ 20 Plasma Immunodepletion Kit (PROT-20)

- Columns, 3 each
Containing 0.3 mL of resin for depletion of 20 high abundance proteins from 8 μ L of human plasma.
- Equilibration Buffer (10 x Concentrate)
- Elution Buffer (10 x Concentrate)
- Kathon (for long term column storage)
- Collection Tubes
- Spin Filters (0.2 μ m for plasma clarification)
- Spin Filters (5,000 NMWL for concentration)
- Syringes (for column equilibration and elution)
- Luer Loc Caps

Depletion Workflow



Methods

Two-Dimensional Electrophoresis (2DE)

Whole citrated plasma samples or depleted plasma (using PROT-20 or another commercially available kit) were diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using PROT-RA (Tributylphosphine and Iodoacetamide). IPG strips (Product Code I3531, 11 cm, pH 4–7) were rehydrated with the samples and focused overnight (85,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Product Code I7281) and loaded onto 8–16% SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170 V for 1.5 h. The marker lanes contain SigmaMarker Wide Range (Product Code M4038). The second dimension gel was fixed and stained with EZBlue (Product Code G1041). The gels were imaged using a Fluor-S™ Multimager (BioRad). The gel images were analyzed using Phoretix 2D Expression software from Nonlinear Dynamics.

High Abundance Protein Depletion

Six (6) high abundance proteins were depleted from fresh citrated plasma using a commercially available product, according to supplied protocols. Twenty (20) high abundance proteins were depleted from plasma using the ProteoPrep® 20 Plasma Immunodepletion Kit (Sigma Product PROT-20). Concentration of multiple depletions was carried out by precipitation (Sigma Product PROT-PR) or using 5,000 NMWL filters (Sigma Product M0286).

ELISA for High Abundance Plasma Proteins

The percent depletion of human plasma proteins was determined by ELISA. Whole citrated plasma and depleted plasma samples were directly coated onto 96-well ELISA plates overnight following dilution in carbonate buffer (Product Code C3041). The plates were washed with TBS-Tween 20 and then incubated with 20 primary antibodies in TBS-BSA for 2 h at 37 °C. The plates were washed and then incubated with HRP-conjugated secondary antibodies in TBS-BSA for 2 h at 37 °C. Finally, the plates were washed and developed with TMB substrate (Product Code T0440), stopped with an equal volume of 1 M HCl and the absorption measured at 450 nm.

Results

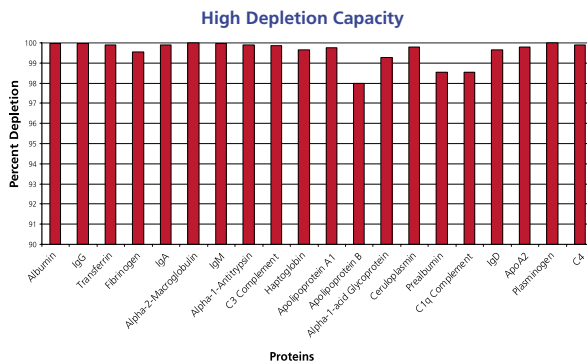


Figure 1: The novel antibody resin (Product Code PROT-20) displays high binding capacity for 20 human plasma proteins.

- Twenty high abundance proteins were removed with an average depletion of 99.6% when 10 x 8 μ L plasma depletions are concentrated and depleted a second time.
 - The average depletion of the twenty proteins from the initial depletions is approximately 95% (data not shown).
 - Depleting the plasma twice significantly improves the efficiency of depleting the 20 proteins.
- Ten (10) initial depletions of 8 μ L were carried out as described in the workflow for use of ProteoPrep 20. The depleted plasma from 10 depletions was pooled and concentrated using a 5,000 NMWL filter. The concentrate was then depleted a second time. An ELISA assay was carried out on all 20 proteins as described in the Methods section.

Unmasking and Increased Loading Capacity

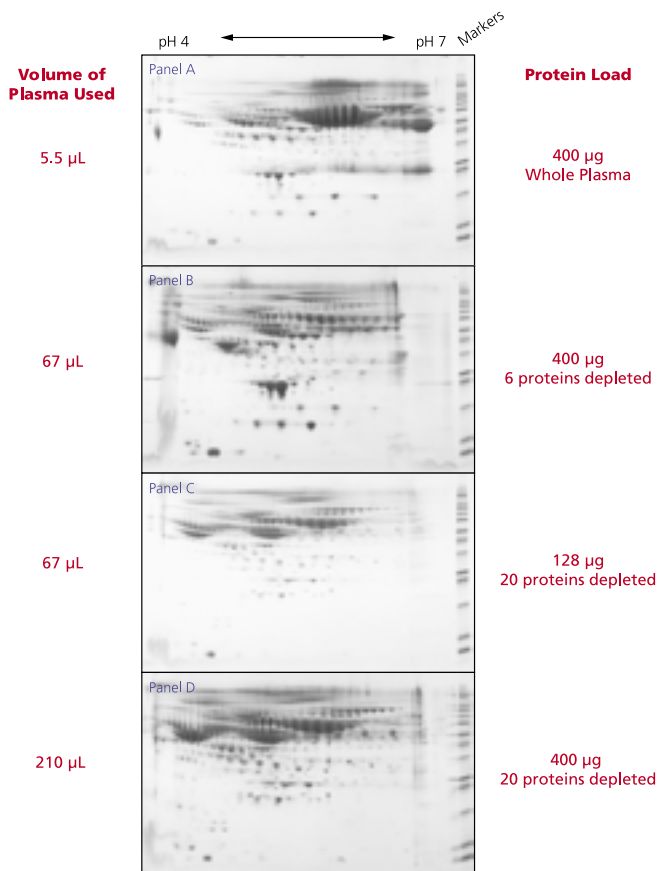
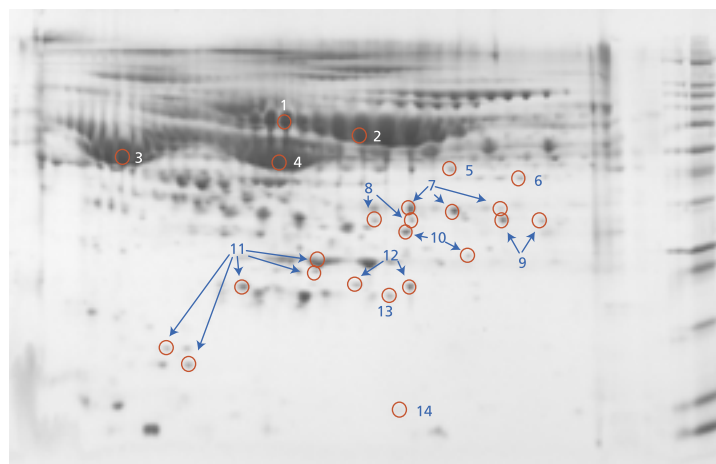


Figure 2: Depletion of 20 proteins allows for higher loads and visualization of low abundance proteins.

- Depletion of 20 vs. 6 proteins from equal volumes of plasma unmasks more comigrating low abundance proteins (Panel C vs. Panel B).
- Depletion of 20 proteins allows for a 3-fold increase in the load of low abundance proteins compared with depletion of just 6 proteins (Panel C vs. Panel D).
- Depletion of 20 proteins allows for a 38-fold increase in the load of low abundance proteins compared with whole plasma (undepleted) (Panel A vs. Panel D).

Samples of whole plasma (Panel A), six protein depleted plasma (Panel B) and PROT-20 depleted plasma (Panels C and D) were concentrated using 5,000 NMWL spin filters as described in the Methods section. Two-dimensional electrophoresis was carried out on all 4 samples as described in the Methods section. Protein concentration was determined by BCA Assay (Product Code QPBCA). The original plasma volume for 400 µg of each sample was 5.5 µL for whole plasma, 67 µL for 6 protein depletion and 67 or 210 µL for PROT-20 depletion.

Mass Spectrometric ID of Proteins Following PROT-20 Depletion



Spot Number	Protein Identification
1	Alpha-1-B-Glycoprotein
2	Hemopexin (Beta-1B-Glycoprotein)
3	Alpha-2-HS-Glycoprotein
4	Vitamin D Binding Protein
5	Pigment Epithelium-Derived Factor
6	Carboxypeptidase N (Kininase 1)
7	Complement Factor H Fragment
8	Apolipoprotein A-IV
9	Ficolin 3 (Hakata Antigen)
10	PK-120 (Plasma Kallikrein-Sensitive Glycoprotein)
11	Apolipoprotein A-IV
12	Glutathione Peroxidase 3
13	Tetranectin (Plasminogen Binding Protein)
14	Serum Amyloid A (SAA)

Figure 3: Low abundance proteins were identified following PROT-20 depletion which were not visualized from either 400 µg of whole plasma or following depletion of 6 proteins.

- Ten (10) low abundance proteins were identified following depletion of 20 proteins.
- Four higher abundance proteins were also identified.
- Several of these proteins were not seen on the gel following depletion of 6 proteins.

Twenty-four (24) spots were excised from the 2DE gel following Coomassie Blue staining of the gel using EZBlue Gel Stain (Product Code G1041). The protein spots were typically digested using a Trypsin Profile IGD Kit (Product Code PP0100). The digests from each spot were analyzed by MALDI TOF MS (Kratos Axima CFR plus). Protein identification was performed using the MASCOT database search algorithm at <http://www.matrixscience.com>.

Conclusions

- Depletion of 20 high abundance proteins from human plasma greatly improves the ability to visualize lower abundance proteins, which may be masked by these 20 proteins.
- This novel antibody resin (0.3 mL) displays high depletion capability (average 99%) for the 20 proteins from 8 µL of human plasma.
- This resin can be reused multiple times (>100 times).
- Depletion of 20 high abundance proteins permits greater loading capacity and visualization of low abundance proteins for electrophoretic and/or chromatographic separation prior to mass spectrometry.

References

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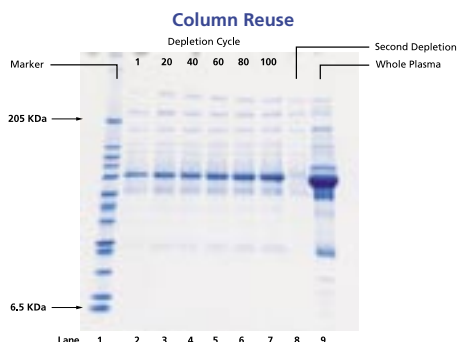


Figure 4: The PROT-20 columns can be reused multiple times.

- Displayed is a SDS-PAGE gel of depleted plasma vs. whole plasma.
- The gel demonstrates that the columns can be reused over 100 times with only a slight drop in depletion performance.
- Pooling the depleted plasma from depletions 93–99 and then carrying out a final depletion demonstrates a significant improvement in the level of depletion (Lane 8).

A sample (volume normalized to 0.3 µL of whole plasma) of depleted plasma from depletions 1, 20, 40, 60, 80 and 100 were run in Lanes 2–7 on a 4–20% SDS-PAGE gel. Also run was a sample from the pooled depletions 93–99 which were concentrated and depleted a final time (Lane 8). Lane 1 contains molecular weight markers and lane 9 contains whole plasma (0.3 µL).



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