

Specific Depletion of Twenty High Abundance Proteins from Human Plasma

M. Schuchard, C. Melm, A. Crawford, H. Chapman, S. Cockrill, K. Ray, R. Mehig, D. Chen, and G. Scott

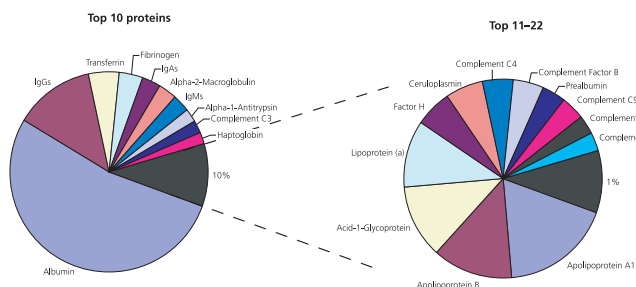
Abstract

Identification of disease biomarkers has significantly increased the interest for study of the human plasma proteome. Unfortunately, disease biomarkers often appear at low concentrations. The plasma proteome has a large dynamic range of individual protein concentrations (10 orders of magnitude); therefore, identification of low copy number proteins of interest is difficult due to the confounding presence of higher abundance proteins. The ProteoPrep® 20 antibody-based resin has been developed to help address these problems by depleting 97–98% of total protein from plasma. This technology removes more proteins than any other comparable product currently available. Depletion of these high abundance proteins allows for visualization of proteins comigrating with, and masked by, the high abundance proteins and peptides, using methods such as LC and 2DE gels. Secondly, depletion allows for individual proteins to be loaded at higher levels for improved visualization/detection of lower abundance proteins. Removing 20 of the most abundant proteins from serum and/or plasma leads to the unmasking of more low copy number proteins and enables loading and detection of more proteins of interest. These combined effects will potentially fuel the discovery of proteins of biological or medical significance.

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 abundant 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- to 50-fold more of each individual protein for improved visualization of lower copy number proteins.

Plasma Facts



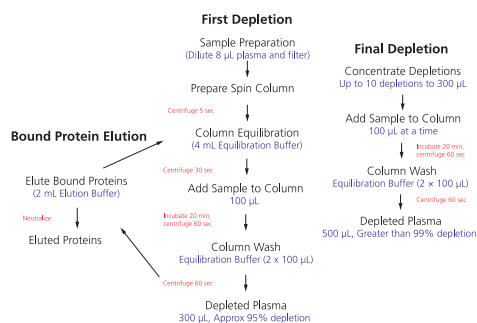
- The 10 most abundant proteins represent approximately 90% of the total protein mass in human plasma.
- The 22 most abundant proteins are said to represent approximately 99% of the total protein mass in human plasma.
- The PROT20 column removes the 20 high abundance plasma/serum proteins listed below. 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

ProteoPrep® 20 Plasma Immunodepletion Kit (PROT20)

- 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 \times Concentrate)
- Elution Buffer (10 \times Concentrate)
- Kathon (for long-term column storage)
- Collection Tubes
- Spin Filters (0.2 μ m for plasma clarification)
- Spin Filters (5000 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 PROT20 or another commercially available kit) were diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using PROTRA (Tributylphosphine and Iodoacetamide). IPG strips (Cat. No. 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 (Cat. No. 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 (Cat. No. M4038). The second dimension gels were fixed and stained with EZBlue™ (Cat. No. G1041). One gel was also silver-stained with PROTSIL2. The gels were imaged using a PowerLook 2100XL scanner (UMAX). The gel images were analyzed using Phoretix 2D Expression software from Nonlinear Dynamics.

High Abundance Protein Depletion

Six high abundance proteins were depleted from fresh citrated plasma using a commercially available product, according to supplied protocols. Twenty high abundance proteins were depleted from plasma using the ProteoPrep® 20 Plasma Immunodepletion Kit (Cat. No. PROT20). Concentration of multiple depletions was carried out by precipitation (Cat. No. PROTBR) or using 5000 NMWL filters (Cat. No. M0286).

Acetone Precipitation and Tryptic Digestion

To one volume of the eluted bound protein fraction five volumes of 100% acetone was added and the samples incubated overnight at –20 °C. The protein was pelleted by centrifugation, and the protein pellet washed and centrifuged 3 times with 50% acetone (–20 °C). The washed protein pellet was air dried at room temperature. The pellet was dissolved with 40 mM ammonium bicarbonate, 9% acetonitrile, pH 8.2, and the proteins were reduced and alkylated (Cat. No. PROTRA). The protein solution was digested with Trypsin (Cat. No. T6567) at a concentration of 1% (w/w) and allowed to incubate at 37 °C for 3 h. Trypsin was again added (1% w/w) and allowed to incubate at 37 °C overnight.

Results

Unmasking and Increased Loading Capacity

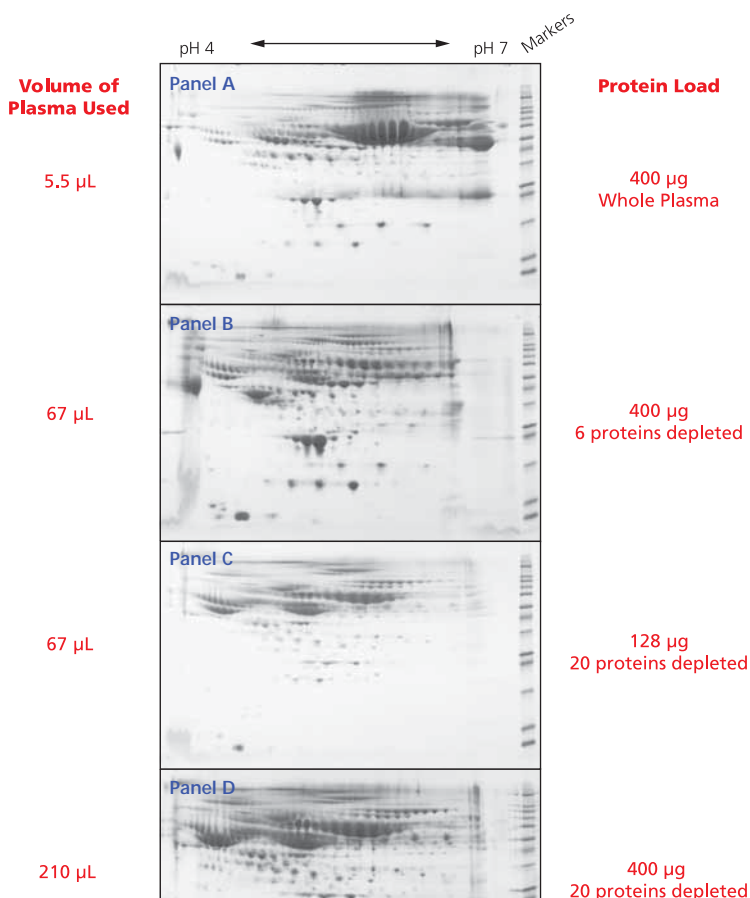
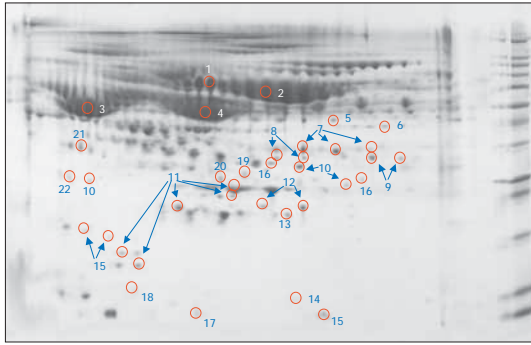


Figure 1: Depletion of 20 proteins allows for higher loads and visualization of low abundance proteins.

- 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 PROT20 depleted plasma (Panels C and D) were concentrated using 5000 NMWL spin filters as described in the Methods section. Two-dimensional electrophoresis was carried out on all four samples as described in the Methods section. Protein concentration was determined by BCA Assay (Cat. No. OPBCA). The original plasma volume for 400 μ g of each sample was 5.5 μ L for whole plasma, 67 μ L for 6 protein depletion, and 67 or 210 μ L for PROT20 depletion.

ID of Proteins Following PROT20 Depletion



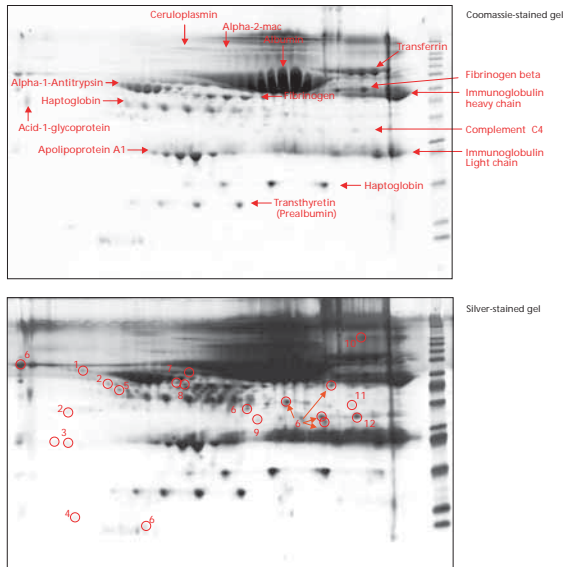
Spot No.	Protein Identification	Spot No.	Protein Identification
1	Alpha-1-B-glycoprotein	12	Glutathione Peroxidase 3
2	Hemopexin (Beta-1B-glycoprotein)	13	Tetranectin (Plasminogen Binding Protein)
3	Alpha-2-HS-glycoprotein (Fetuin A)	14	Serum amyloid A (SAA)
4	Vitamin D Binding Protein	15	Kininogen
5	Pigment Epithelium-derived factor	16	Complement Factor H-related protein 2
6	Carboxypeptidase N (Kininase 1)	17	Inter-alpha-trypsin inhibitor H1
7	Complement Factor H fragment	18	Vitronectin
8	Apolipoprotein E	19	Gelatin-Binding Protein
9	Ficolin 3 (Hakata antigen)	20	Antithrombin III
10	PK-120 (Plasma Kallikrein-sensitive Glycoprotein)	21	Clusterin (Apolipoprotein J)
11	Apolipoprotein A-IV	22	Inter-alpha-trypsin inhibitor H4

Figure 2: Low abundance proteins were identified following PROT20 depletion which were not visualized from either 400 µg of whole plasma or following depletion of 6 proteins.

- Eighteen low abundance proteins were identified following depletion of 20 proteins.
- Four higher abundance proteins were identified.
- Several of these proteins were not seen on the gel following depletion of six proteins.

Thirty-five spots were excised from the 2DE gel following Coomassie Blue staining of the gel using EZBlue™ Gel Stain (Cat. No. G1041). The protein spots were trypsin digested using a Trypsin Profile In Gel Digestion Kit (Cat. No. PPO100). The digests from each spot were submitted for MALDI-TOF MS analysis. Protein identification was performed using the MASCOT database search algorithm at www.matrixscience.com.

Mass Spectrometric ID of Bound Proteins



Spot No.	Protein Identification	Spot No.	Protein Identification
1	Alpha-1-antichymotrypsin	7	IgA-2 heavy chain
2	Alpha-1-antitrypsin	8	Fibrinogen gamma chain
3	Immunoglobulin J chain	9	Complement C3
4	Apolipoprotein CIII	10	Transferrin
5	Serum Paraoxonase	11	Cytokeratin
6	Albumin	12	Complement C4

Figure 3: Bound proteins were identified following PROT20 depletion. The novel antibody resin displays high specificity.

- Of the spots identified, all but two were fragments of, or related to the 20 specifically depleted proteins.
- Alpha-1-antichymotrypsin has no apparent relationship to the 20 proteins.
- Cytokeratin was likely a contaminant during processing.

Bound proteins eluted from the resin were acetone precipitated. 2DE was run on the precipitated protein pellet. The gel was first Coomassie-stained and then silver-stained (Cat. No. PROTSIL2). Spots (21) were excised from the 2DE gel following Coomassie- or silver-staining of the gel using PROTSIL2. The protein spots were trypsin-digested using a Trypsin Profile In Gel Digestion Kit (Cat. No. PPO100). The digests from each spot were submitted for MALDI-TOF MS analysis. Protein identification was performed using the MASCOT database search algorithm at www.matrixscience.com.

Specifically Bound Proteins

Reference	XC Score	No. of peptides	Coverage DeltaCn
1 ALBU_HUMAN (P02768) Serum albumin precursor	230	23	29
2 IGHG1_HUMAN (P01857) Ig gamma-1 chain C region	60	6	25
2 IGHG2_HUMAN (P01859) Ig gamma-2 chain C region	50	5	22
2 IGHG3_HUMAN (P01860) Ig gamma-3 chain C region	46	5	16
2 IGHG4_HUMAN (P01861) Ig gamma-4 chain C region	40	4	18
3 TRFE_HUMAN (P02787) Serotransferrin precursor (Transferrin) (Siderophilin)	80	8	13
4 FIBA_HUMAN (P02671) Fibrinogen alpha chain precursor [Contains: Fibrinopeptide A]	210	21	29
4 FIBB_HUMAN (P02675) Fibrinogen beta chain precursor [Contains: Fibrinopeptide B]	140	14	40
4 FIBG_HUMAN (P02679) Fibrinogen gamma chain precursor	110	11	37
5 IGH1A1_HUMAN (P01876) Ig alpha-1 chain C region	70	7	21
5 IGH2A2_HUMAN (P01877) Ig alpha-2 chain C region	50	5	15
6 A2MG_HUMAN (P01023) Alpha-2-macroglobulin precursor (Alpha-2-M)	280	28	28
7 MUC_HUMAN (P01871) Ig mu chain C region	60	6	15
8 A1AT_HUMAN (P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor)	160	16	47
9 CO3_HUMAN (P01024) Complement C3 precursor	510	51	41
10 HPT_HUMAN (P00738) Haptoglobin precursor	78	8	21
10 HPTR_HUMAN (P00739) Haptoglobin-related protein precursor	38	4	10
11 APOA1_HUMAN (P02647) Apolipoprotein A-I precursor (Apo-AI)	90	9	37
12 APOA2_HUMAN (P02652) Apolipoprotein A-II precursor (Apo-AII)	30	3	32
13 APOB			
14 A1AG1_HUMAN (P02763) Alpha-1-acid glycoprotein 1 precursor (AGP 1) (Orosomucoid 1)	30	3	16
14 A1AG2_HUMAN (P19652) Alpha-1-acid glycoprotein 2 precursor (AGP 2) (Orosomucoid 2)	30	3	13
15 CERU_HUMAN (P00450) Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase)	120	12	19
16 CO4_HUMAN (P01028) Complement C4 precursor	170	17	15
17 C1QB_HUMAN (P02746) Complement C1q subcomponent, B chain precursor	10	1	
17 C1QC_HUMAN (P02747) Complement C1q subcomponent, C chain precursor	10	1	
18 IGHJ			
19 THY_HUMAN (P02766) Transthyretin precursor (Prealbumin)	60	6	68
20 PLMN_HUMAN (P00747) Plasminogen precursor	20	2	4

Nonspecifically Bound Proteins

Reference	XC Score	No. of peptides	Coverage DeltaCn
HBA_HUMAN (P69905) Hemoglobin alpha chain	30	3	31
HBB_HUMAN (P68871) Hemoglobin beta chain	50	5	38
HBD_HUMAN (P02042) Hemoglobin delta chain	30	3	24
HPTR_HUMAN (P00739) Haptoglobin-related protein precursor	38	4	10
PZP_HUMAN (P20742) Pregnancy zone protein precursor	20	2	2
PON1_HUMAN (P27169) Serum paraoxonase/arylesterase 1	10	1	6
METE_MET1A (Q58868) Probable methylcobalamin-homocysteine methyltransferase	10	1	5
ATP4A_HUMAN (P20648) Potassium-transporting ATPase alpha chain 1	10	1	2
BIRC2_HUMAN (Q13490) Baculoviral IAP repeat-containing protein 2	8	1	5
KIR3_HUMAN (Q8IU9) Kin of IRRE-like protein 3 precursor	8	1	3
Y483_MYCPN (P75302) Hypothetical protein MG335.2 homolog	10	1	8
FA57A_HUMAN (Q8TBR7) Protein FAM57A (CT120 protein)	6	1	5
NEIL1_HUMAN (Q96F14) Endonuclease VIII-like 1	4	1	4
CENG3_HUMAN (Q96P47) Centaurin-gamma 3	10	1	3
EPHA1_HUMAN (P21709) Ephrin type-A receptor 1 precursor	10	1	1
UBP22_HUMAN (Q9UPT9) Ubiquitin carboxyl-terminal hydrolase 22	10	1	3

Figure 4: Bound proteins from PROT20 were evaluated using LC-MS/MS.

- Of the 20 specifically bound proteins, 18 were positively identified. Apolipoprotein B and IgD were not specifically detected.
- Three (3) non-specifically bound proteins were identified with 2 or more peptides.
- Eleven (11) non-specifically bound proteins were identified with 1 peptide.

Bound proteins eluted from the PROT20 resin were acetone-precipitated and trypsin-digested as indicated in *Methods.* A sample was injected onto a C18 column and separated with a 200-minute acetonitrile gradient and the peptides identified via LC-MS/MS (LTO, Thermo).

Affinity Capture Criteria

Advantages	<ul style="list-style-type: none"> • 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. • Greater loading of low abundance proteins for electrophoretic and/or chromatographic separation prior to mass spectrometric analysis. • High depletion capability (average 99%) for the 20 proteins from 8 µL of human plasma. • Reusable a minimum of 100 times (data not shown).
Methodology	Resin bioconjugated to 20 antibodies via linkages designed to reduce non-specific binding.
Mono/polyclonal	Antibodies consist of both polyclonal IgGs and single-chain antibodies.
Throughput	This platform will initially be offered in a spin column format but eventually expanded into high throughput configurations.
Cost	The cost for the 3-column kit will be approximately \$2,500 and will be available in January 2006.
Verification	PROT20 has been successfully verified using the following formats: 1DE, 2DE, Difference Gel Electrophoresis (DIGE), iTRAQ labeling, ELISA, MALDI-TOF Mass Spectrometry, ESI Mass Spectrometry.
Specificity	Low non-specific binding is demonstrated by the relatively few number of bound proteins detected.

References

1. Anderson, N. L. and Anderson, N. G. The Human Plasma Proteome. *Mol. Cell. Proteomics* **2002**, *1*, 845.
2. Adkins, J. N. et al. Toward a Human Blood Serum Proteome. *Mol. Cell. Proteomics* **2002**, *1*, 947.
3. Rengarajan, K. et al. Removal of Albumin from Multiple Human Serum Samples. *BioTechniques* **1996**, *20*, 30.