Novel ProteoPrep® 20 Immunoaffinity Depletion Resin for Human Plasma

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Abstract
We have developed a novel, high-binding capacity, antibody-based resin for the depletion of twenty high abundance proteins in human plasma. These 20 high abundance proteins represent approximately 95% of total protein. This is the first time that a plasma depletion kit has been available that removes 20 high abundance proteins. The technology is a significant improvement over existing kits that remove 2, 6, or 12 proteins. The ProteoPrep® 20 Plasma Immunodepletion Kit is optimized for 8 µl plasma volumes per cycle and is supplied in a convenient spin column format. Depletion of 95% of the total protein from human plasma allows for a 20-fold increase in the load of proteins of interest when compared to whole plasma. This novel technology demonstrates the ability to deplete more proteins from more plasma, thus allowing researchers greater ability to visualize/analyze 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 huge importance, especially for the pharmaceutical potential of identifying disease biomarkers. Many proteins of 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. To address these issues, an affinity resin has been developed for removal of 20 high abundance proteins from 8 µl of plasma. Depletion of these abundant proteins allows for visualization of proteins that co-migrate with, and are masked by, the high abundance proteins during 1-D or 2-D gel electrophoresis and HPLC separations. Plasma proteins can then be loaded onto gels, IPG strips, or HPLC columns at higher levels for improved visualization/detection of low copy number proteins.

Plasma facts
A challenge for studying the human plasma proteome is its wide range of protein concentrations ranging over 10 orders of magnitude. In particular, low concentration proteins are masked by high abundance proteins. For example, in plasma approximately 35-50 mg/ml of albumin is present, along with 10 pg/ml of interleukin 6 (a sensitive indicator of inflammation or infection with a molecular weight of 21 kDa). The interleukin 6 abundance is almost 10 orders of magnitude less than albumin.

The 10 most abundant proteins in human plasma represent approximately 90% of the total protein mass; the next 12 abundant proteins are said to make up to about 9% of total protein mass (Figure 1). Therefore, approximately 99% of the total protein mass in human plasma consists of the 22 most abundant proteins.

Next to albumin, 21 additional proteins are depicted in Figure 1 that result in 99% of the total plasma protein amount, leaving 1% for an estimated 10,000 proteins – at very low concentrations. Most of the proteins of pharmaceutical interest appear in this last 1% [1].

The ProteoPrep® 20 technology removes the 20 high abundance plasma/serum proteins listed in Table 1. These 20 proteins represents 95% of the total human plasma protein mass.

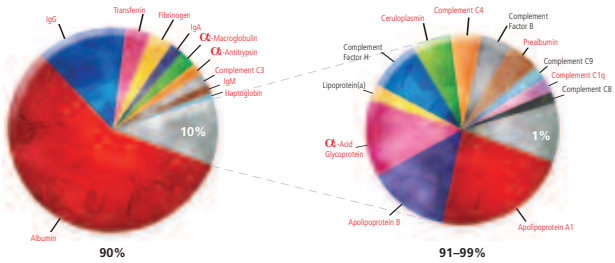


Figure 1. *Relative abundance of proteins in human plasma.*

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

Table 1. *Twenty high abundance proteins in human plasma removed from human plasma by the ProteoPrep® 20 technology.*

ProteoPrep® 20 Plasma Immunodepletion Kit (PROT20 and PROT20S)

The ProteoPrep® 20 Plasma Immunodepletion Kit contains the following items:

- Columns 3 or 1, respectively, for the PROT20 and PROT20S (each column containing 0.3 ml of resin for depletion of 20 high abundance proteins from 8 µl of human plasma)
- ProteoPrep® 20 Equilibration Buffer (10x concentrate)
- ProteoPrep® 20 Elution Buffer (10x concentrate)
- ProteoPrep® 20 Preservative Concentrate (for long-term column storage)
- Collection tubes
- Spin Filters (0.2 µm for plasma clarification)
- Spin Filters (5,000 NMWL for concentration)
- Syringes (for column equilibration and elution)
- Luer Loc

Methods

Depletion of plasma samples

According to the workflow delineated in Figure 2, the column is first washed with equilibration buffer and briefly centrifuged to remove excess buffer and reduce sample dilution. Next, 8 µl of human plasma (or serum) is diluted to 100 µl with equilibration buffer. The diluted plasma is applied to the semi-dry resin bed and allowed to incubate for 15-20 minutes. The depleted plasma and two subsequent column washes (100 µl each) are then microcentrifuged from the column. The depleted plasma (300 µl) has approximately 95% of the 20 high abundance proteins removed. The column is now stripped of the bound proteins using 2 ml of elution buffer and re-equilibrated for another depletion cycle. The diluted and depleted plasma from up to 10 depletion cycles (80 µl of total plasma) is concentrated to 200-300 µl using 5,000 NMWL ultrafiltration spin columns and re-applied to the column for a final or "polishing" (11th) depletion. The final depleted plasma from 80 µl of original plasma contains less than 1% of the 20 depleted proteins.

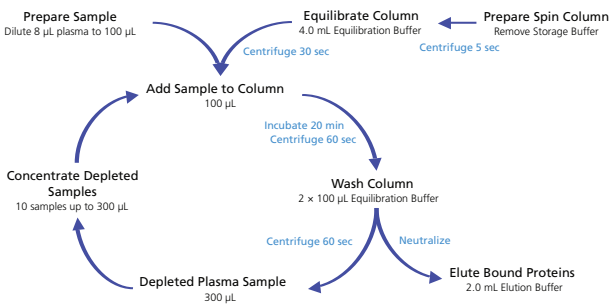


Figure 2. *Depletion workflow.*

Two-dimensional electrophoresis (2-DE)

Using PROT20 or another commercially available kit capable of depleting 6 high abundant proteins, whole citrated plasma samples or depleted plasma were diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using PROTRA (Tributylphosphine and Iodoacetamide). IPG strips (11 cm, pH 4-7) were rehydrated with the samples and focused overnight (85,000 Vhr). The strips were equilibrated for 15 minutes with IPG Equilibration Buffer and loaded onto 8-16% SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170 V for 1.5 hours. The marker lanes contained SigmaMarker™ Wide Range. The second dimension gel was fixed and stained with EZBlue™. The gels were imaged using a Fluor-S™ MultiImager (BioRad). 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. 20 high abundance proteins were depleted from plasma using the ProteoPrep® 20 Plasma Immunodepletion Kit. Concentration of multiple depletions was carried out by precipitation or by using 5,000 NMWL filters.

ELISA for high abundance plasma proteins

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

Trypsin digestion and LC MS/MS

The samples were reduced and alkylated using the PROTRA (Tributylphosphine and Iodoacetamide) and digested with Trypsin at a concentration of 1% (w/w) and allowed to incubate at 37 °C for 3 hours. Trypsin was again added (1% w/w) and the samples allowed to incubate at 37 °C overnight to ensure complete digestion. The digests were dried in a vacuum centrifuge and dissolved with 40 µl of 0.1% TFA. Digested samples (8 µl, 76 µg total protein) were separated by reverse phase on a 150 x 2.1 mm, 5 µm Supelco Discovery™ HS C18 column. The separation was performed over 3 hours with a gradient of formic acidified water and acetonitrile. Tandem mass spectrometric data was acquired using a Thermo Finnegan LTQ via a Data Dependent Acquisition (DDA) method with dynamic exclusion. Data sets were searched using the SEQUEST algorithm on Bioworks 3.2.

Results and Discussion

High depletion capacity

The novel antibody resin of the ProteoPrep® 20 Plasma Immunodepletion Kit displays high binding capacity for 20 human plasma proteins by ELISA (Figure 3). The 20 high

abundance proteins were removed with an average depletion of 99.3% when ten sequential 8 µl plasma depletions were concentrated and depleted a second time. Depleting the plasma twice significantly improves the efficiency of depleting the 20 proteins. The average depletion of the 20 proteins from the initial depletions was approximately 95% (data not shown).

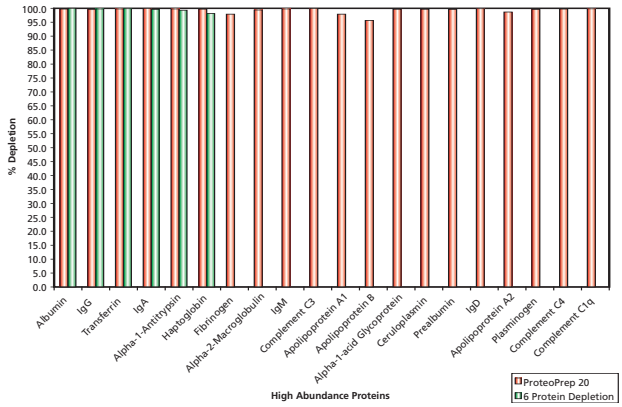


Figure 3. *High depletion capacity. The percentage of depletion by each antibody used in the ProteoPrep® 20 Plasma Immunodepletion Kit and the Agilent product is depicted above. 20 high abundance proteins were removed with an average depletion of 99.3% when plasma depletions were concentrated and depleted a second time. Six high abundance proteins were removed with an average depletion of 99.4% when plasma was depleted similarly using the Agilent product. The total amount of protein removed using these two technologies was approximately 94% (ProteoPrep® 20) and 81% (6 protein depletion), as determined by Bradford Assay. The depleted plasma from both depletions was pooled and concentrated using a 5,000 NMWL filter. An ELISA assay was carried out on all 20 proteins as described in the Methods section.*

Unmasking and increased loading capacity

Increased gel loading capacity has been investigated. Specifically, the depletion of 20 high abundance proteins (ProteoPrep® 20 Plasma Immunodepletion Kit) has been compared with the depletion of 6 high abundance proteins (Agilent cat. no. 5188-5230), and non-depleted plasma. In Figure 4 non-depleted plasma, 5.5 µl corresponding to 400 µg of total protein load, was 2-D electrophoretically separated as a control (Panel A). The presence of high abundance proteins resulted in blurry and indistinct spots. Secondly, the depletion of 6 proteins was investigated using a 2-D gel (Panel B). Following depletion, a plasma volume of 67 µl, instead of 5.5 µl (corresponded to a 400 µg of protein load), enabled a 12-times higher serum volume load after depletion. Faint spots in the gel of panel A became more visible and unmasked after depletion. In addition the same initial plasma volume of 67 µl was depleted using the ProteoPrep® 20 Plasma Immunodepletion Kit (Panel C). The 67 µl volume of plasma corresponded to only 128 µg of protein due to the effective depletion of the 20 high abundant proteins. Clearly depletion unmasked significantly more co-migrating low abundance proteins (Panel C vs. Panel B). Finally the same protein load of 400 µg was 2-D separated after depletion of 210 µl of plasma using the ProteoPrep® 20 Plasma Immunodepletion Kit (Panel D). Many new, clearly separated spots became observable and available for identification by mass spectrometry.

Consequently the depletion of 20 proteins allowed for a 3-fold increase in the load of low abundance proteins compared with depletion of just 6 proteins (Panel C vs. Panel D). When compared against whole plasma (undepleted), the depletion of 20 proteins allowed for a 38-fold increase in the load of low abundance proteins (Panel A vs. Panel D).

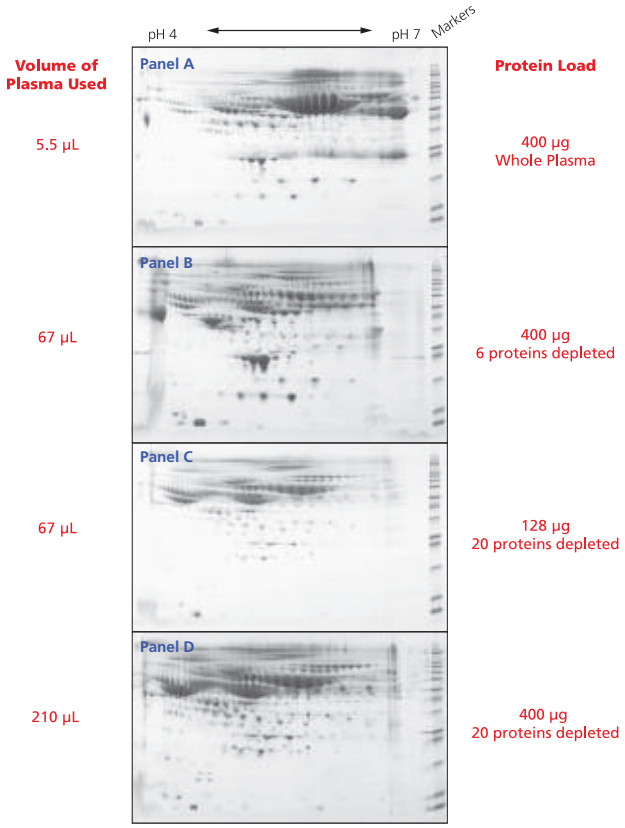


Figure 4. *Depletion of 20 proteins allows for higher loads and visualization of low abundance proteins. Samples of whole plasma (Panel A), six protein depleted plasma (Panel B) and PROT20 depleted plasma (Panels C and D) were concentrated using 5,000 NMWL spin filters as described in the Methods section. Two-dimensional electrophoresis was carried out on all 4 samples as described in the Methods section. The protein concentration was determined by BCA assay. The original plasma volume for 400 µg of each sample was 5.5 µl for whole (undepleted) plasma, 67 µl for 6 protein depletion, and 67 µl or 210 µl for PROT20 depletion.*

Mass spectrometric ID of proteins following PROT20 depletion

The identification of proteins in the non-depleted, 6 protein depleted, and 20 protein depleted plasmas was performed using so-called "bottom-up" mass spectrometry. ProteoPrep® 20 depleted plasma, 6 protein depleted plasma and whole plasma (380 µg) were trypsin digested overnight and dried using a vacuum centrifuge. The samples were prepared as described in the Methods section. Protein identifications were made via SEQUEST search and filtered as follows; Xcorr values ≥ 1.9, 2.4, and 2.9 for singly, doubly, and triply charged ions, with delta CN value ≥ 0.1, and protein probability of 99.9%. Panel A lists the lower abundance proteins identified in the three samples with the number of different peptides detected and their sequence coverage. The 20

higher abundance proteins are not listed. Panel B is a graph of the average sequence coverage for the 16 proteins in Panel A, which were identified in all three samples. The Venn Diagram (Panel C) illustrates the overlap of the three datasets.

Mass spectrometric ID of bound proteins

In addition to the proteins in the depleted fraction, the bound proteins have also been investigated. For that purpose, the bound protein fraction was eluted and electrophetically separated (Figure 6). The six proteins detected are listed in the table and annotations have been made on the gels in Figure 6. From the spots identified, all but two were fragments of, or related to, the 20 specifically depleted proteins: Alpha-1-antichymotrypsin is known to have high sequence homology with Alpha-1-antitrypsin, one of the 20 depleted proteins. Serum Paraoxonase is known to be associated with

Panel A – Bottom Up Analysis

	PROT20 Depleted		6 Protein Depleted		Whole Plasma	
Protein Identification	Peptide	% Coverage	Peptide	% Coverage	Peptide	% Coverage
Fibronectin precursor	27	16	18	11	4	2
Alpha-2-HS-glycoprotein precursor, Fetuin-A	22	31	7	28	2	6
Vitamin D-binding protein precursor	18	36	15	31	7	17
Apolipoprotein A-IV precursor	18	43	9	28	1	4
Inter-alpha-trypsin inhibitor heavy chain H2 precursor	17	17	8	13	2	4
Hemopexin precursor	16	28	11	18	10	25
Alpha-1B-glycoprotein precursor	11	24	11	23	3	8
Plasma protease C1 inhibitor precursor	11	15	4	11	7	10
Inter-alpha-trypsin inhibitor heavy chain H4 precursor	10	13	5	7	1	1
Afamin precursor	10	14	2	4	1	3
Beta-2-glycoprotein I precursor, Apo-H	8	19	8	26	6	16
Alpha-1-antichymotrypsin precursor	7	16	4	12	2	7
Angiotensinogen precursor	5	16	5	17	1	4
Carboxypeptidase N subunit 2 precursor	4	7	3	7	2	9
Vitronectin precursor	4	12	2	6	3	7
AMBP protein precursor	4	10	1	3	1	3
Complement factor B precursor	17	19	10	14		
Kininogen-1 precursor	14	18	12	18		
Gelsolin precursor	14	20	7	11		
Complement factor H precursor	13	11	5	4		
Prothrombin precursor	13	21	3	6		
Inter-alpha-trypsin inhibitor heavy chain H1 precursor	10	13	4	7		
Pigment epithelium-derived factor precursor	8	16	6	11		
Histidine-rich glycoprotein precursor	5	10	2	5		
Serum amyloid P-component precursor	4	17	5	24		
Clusterin precursor	4	10	3	11		
Heparin cofactor II precursor	4	7	2	4		
Complement component C9 precursor	3	6	1	2		
Serum paraoxonase/arylesterase 1	2	7	3	9		
C4b-binding protein alpha chain precursor	2	2	2	2		
Complement component C8 gamma chain precursor	2	13	1	7		
Complement factor I precursor	2	4	1	2		
Adapter-related protein complex 4 beta 1 subunit	1	2	3	5		
Sex hormone-binding globulin precursor	1	6	1	6		
Plasma retinol-binding protein precursor	1	5	1	5		
Complement C1s subcomponent precursor	1	2	1	2		
Antithrombin-III precursor	8	19				
N-acetylmuramoyl-L-alanine amidase precursor	6	17				
Complement C5 precursor	6	4				
Alpha-2-antiplasmin precursor	4	11				
Zinc-alpha-2-glycoprotein precursor	3	13				
Heat-shock protein beta-7	2	10				
Coagulation factor XII precursor	2	5				
Complement C2 precursor	2	3				
Tetranectin precursor	1	6				
Activator 1 140 kDa subunit	1	2				
Galectin-3 binding protein precursor	1	2				
Kallistatin precursor	1	4				
Complement component C6 precursor	1	2				
Corticosteroid-binding globulin precursor	1	3				
Leucine-rich alpha-2-glycoprotein precursor	1	3				
Nuclear pore complex protein Nup98-Nup96 precursor			2	1	2	1
Apolipoprotein C-III precursor			2	27	2	27
Periplakin			2	2		
Myosin Ic			2	4		
Ninein			1	1		
Probable G-protein coupled receptor 109B			1	7		
Complement factor H-related protein 3 precursor			1	3		
Ankyrin repeat and SOCS box protein 12			1	10		
Neuronal acetylcholine receptor protein			1	4		
Type 2 lactosamine alpha-2,3-sialyltransferase					3	7
Zinc finger protein 205					1	6
Tyrosine-protein kinase BTK					1	3

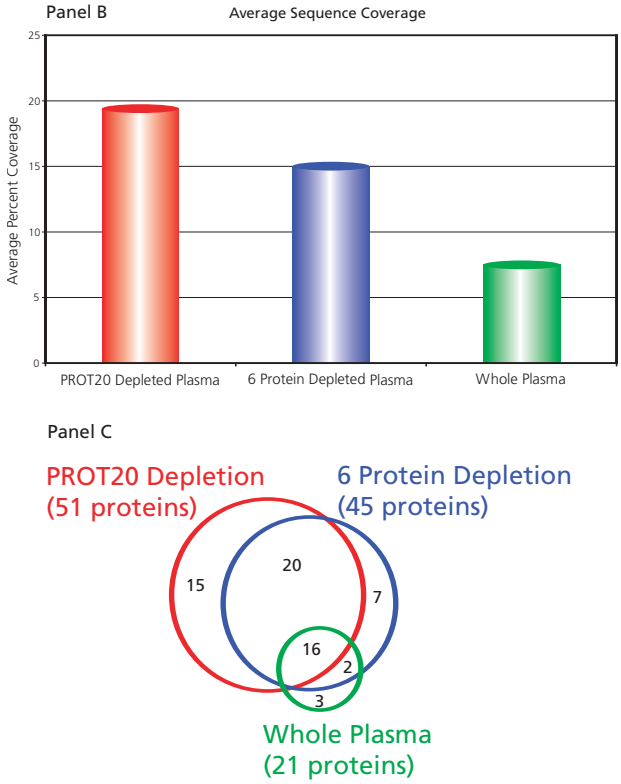


Figure 5. *Bottom up analysis: depletion of more proteins increases the number of protein identifications and allows for increased sequence coverage. The number of proteins identified was higher with the PROT20 depleted sample (Panels A and C). The sequence coverage was generally higher with the PROT20 depleted sample (Panel B).*

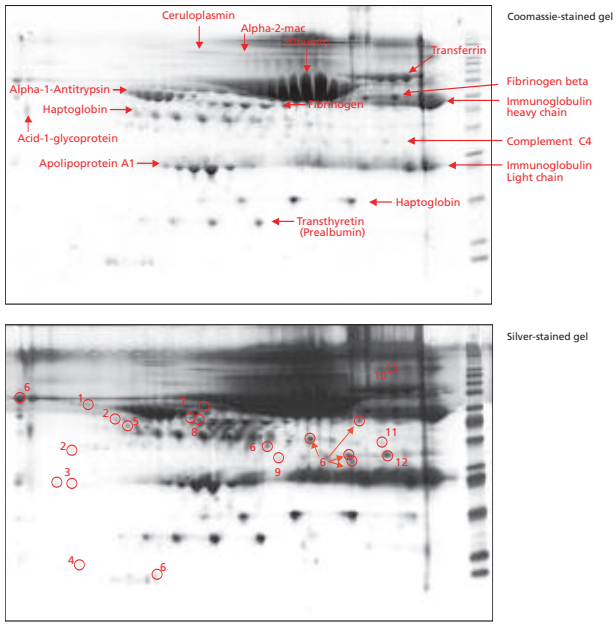
high density lipoproteins and was likely depleted during removal of Apolipoprotein A-I and A-II. Also Cytokeratin was likely a contaminant during processing. Overall, the results obtained indicate a high specificity of the depletion procedure.

Column reuse

Important for the economic evaluation of a kit is its lifetime and reusability. Therefore the performance of the ProteoPrep® 20 Plasma Immunodepletion Kit during 100 depletion repetitions has been analyzed. The columns can be reused at least 100 times with only a slight drop in depletion performance (approximately 5%). If depletions 93-99 are pooled, concentrated and re-depleted, a high degree of depletion is obtained (average >99%).

Summary

This novel ProteoPrep® 20 technology displays high depletion capability (average 99%) for 20 high abundance proteins from 8 µl of human plasma. The spin column format is capable of handling small to medium plasma/serum volumes and reduces dilution to a great extent in order to enable efficient downstream analysis.



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 6. *Bound proteins were identified following PROT20 depletion. Bound proteins eluted from the resin were acetone precipitated. 2-DE was run on the precipitated protein pellet. The gel was first Coomassie-stained and then silver-stained. Twenty-one (21) spots were excised from the 2-DE gel following Coomassie (top) or silver staining (bottom) of the gel using PROTSIL2. The protein spots were trypsin-digested using a Trypsin Profile In Gel Digestion Kit. 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.*

The depletion of 20 high abundance proteins using the ProteoPrep® 20 Plasma Immunodepletion Kit permits greater loading capacity and visualization of low abundance proteins for electrophoretic and/or chromatographic separation prior to mass spectrometric analysis.

The depletion of 20 high abundance proteins from human plasma greatly improves the ability to visualize/detect lower abundance proteins, which were masked by the 20 proteins. The ProteoPrep® 20 Plasma Immunodepletion Kit is of high economic value as each column can be reused multiple times (≥100 times).

References

1. Anderson, N.L. and Anderson, N.G. The Human Plasma Proteome. *Mol. Cell. Proteomics,* **1,** 845-867 (2002).
2. Adkins, J.N., *et al.,* Toward a Human Blood Serum Proteome. *Mol. Cell. Proteomics,* **1,** 947-955 (2002).
3. Omenn, G.S., *et al.,* Overview of the HUPO Plasma Proteome Project: Results from the Pilot Phase with 35 Collaborating Laboratories and Multiple Analytical Groups, Generating a Core Dataset of 3020 Proteins and a Publicly-available Database. *Proteomics,* **5,** 3226-3245 (2005).

Ordering Information

Cat. No.	Description	Unit
PROT20	ProteoPrep® 20 Plasma Immunodepletion Kit	1 kit
PROT20S	ProteoPrep® 20 Plasma Immunodepletion Kit – Single	1 kit
C0356	Protein Extraction Reagent Type 4	4 btl
PROTRA	ProteoPrep® Reduction and Alkylation Kit	1 kit
I3531	IPG strips, 11 cm, pH 4-7	12 ea
I7281	IPG Equilibration Buffer	1 btl
G1041	EZBlue™ Gel Staining Reagent	500 ml 3.8 L

Cat. No.	Description	Unit
M0286	5,000 NMWL Filter	25 ea
PROTPR	ProteoPrep® Protein Precipitation Kit	1 kit
C3041	Carbonate-Bicarbonate Buffer	50 cap 100 cap
T0440	3,3', 5,5' – Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA	100 ml 1 L
QPBCA	QuantiPro BCA Assay Kit	1 kit
B6916	Bradford Reagent	500 ml
PP0100	Trypsin Profile IGD Kit	1 kit
PROTSIL2	ProteoSilver™ Plus Silver Stain Kit	1 kit

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Biomarker discovery is at the forefront of modern day proteomics, and human plasma has become the sample source of choice. Until now, discovering proteins that can be used as biomarkers from such a wide, dynamic range of human plasma, has felt like searching for a needle in a haystack.

Now Sigma is leading the way towards better biomarker discovery through removal of twenty high abundance proteins from human plasma. The segment of the plasma proteome that researchers can now observe is shifting dramatically towards the lower abundance proteins believed to be the frontier of discovery. The ProteoPrep 20 Plasma Immunoaffinity Kit removes 97% of the overall protein load, leading to the visualization of proteins previously not seen.

Through Sigma innovation, biomarker discovery has reached a new level. Researchers are now delving deeper into the plasma proteome and discovering more unique data.

Find out how you can discover more unique proteins by visiting us at:

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