Comparison of Precipitation Methods Following Depletion of Twenty High Abundance Proteins from Human Plasma

M. Schuchard, C. Melm, A. Crawford, H. Chapman, J. Wildsmith, K. Ray, D. Chen, and G. Scott

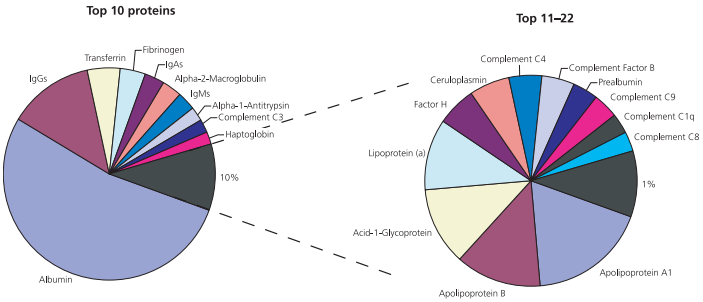
Abstract

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 co-migrating with, and masked by the high abundance proteins and peptides using methods such as LC, LC-MS/MS, and 2DE gels. Secondarily, depletion allows for individual proteins to be loaded at higher levels for improved visualization of lower abundance proteins. Removing twenty of the most abundant proteins from serum and/or plasma, leads to the unmasking of more low copy number proteins and being able to load and detect more proteins of interest. Here we compare three methods of desalting/precipitation following depletion, and evaluate the resulting low abundance protein identifications made via LC-MS/MS.

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 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 µL 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.
- Removal of salts, or concentration, is often required for downstream processing following depletion. Three common methods include buffer exchange using a 5000 NMWL filter, TCA precipitation, or acetone precipitation. We have begun a comparison of plasma protein identifications following depletion and further processing using these methods.

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 represent 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 µL of human plasma)
- Equilibration Buffer (10× Concentrate)
- Elution Buffer (10× Concentrate)
- Kathon (for long-term column storage)
- Collection Tubes
- Spin Filters (0.2 µm for plasma clarification)
- Spin Filters (5000 NMWL for concentration)
- Syringes (for column equilibration and elution)
- Luer Lock Caps

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 (Cat No. C0356) and reduced and alkylated using PROTRA (Tributylphosphine and Iodoacetamide). IPG strips (Cat. No. I3531, 11 cm, pH 4–7) were rehydrated with the samples 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 contained SigmaMarker Wide Range (Cat. No. M4038). The second dimension gels were fixed and stained with EZBlue™ (Cat. No. G1041).

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. PROTPR) or using 5000 NMWL filters (Cat. No. M0286).

Buffer Exchange

The buffer in the PROT20 depleted sample (230 µg protein) from 90 µL of human plasma was exchanged approximately 500-fold with 50 mM ammonium bicarbonate buffer, pH 8.2, in an Amicon Ultra 4 (5000 NMWL filter) and centrifuged at 5000 × g at 2–8 °C. To the sample was added 1/10th volume of acetonitrile.

TCA Precipitation

The protein from a PROT20 depleted sample (230 µg protein) from 90 µL of human plasma was TCA precipitated using the ProteoPrep Protein Precipitation Kit (Cat. No. PROTPR). To the depleted sample was added an equal volume of 0.2 % DOC and TCA (1/10 volume). The sample was stored on ice for a minimum of 30 min and microcentrifuged at 14,000 rpm for 10 min. The pellet was washed 3 times with ice cold Wash Solution (25% acetone) and microcentrifuged for 5 min. The pellet was air dried and dissolved in 50 mM ammonium bicarbonate buffer, pH 8.2, with 9% acetonitrile.

Acetone Precipitation

To the PROT20 depleted sample (230 µg protein) from 90 µL of human plasma was added five volumes of 100% acetone. The sample was incubated at –20 °C overnight. The protein was pelleted by centrifugation and the protein pellets were washed 3 times with 50% acetone (–20 °C) with centrifugation. The washed protein pellet was air dried at room temperature. The pellet was dissolved in 50 mM ammonium bicarbonate, with 9% acetonitrile, pH 8.2.

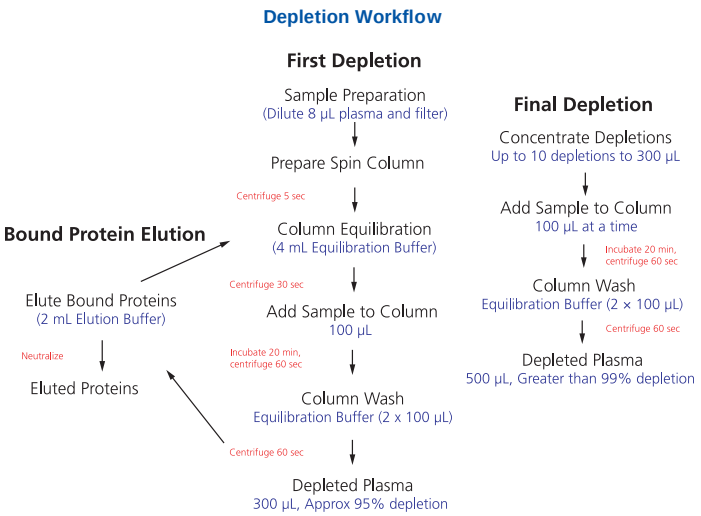
Trypsin Digestion

The samples were reduced and alkylated (Cat. No. PROTRA) and 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 the sample allowed to incubate at 37 °C overnight. The digests were dried down in a Speed Vac and dissolved with 50 µL of 0.1% TFA.

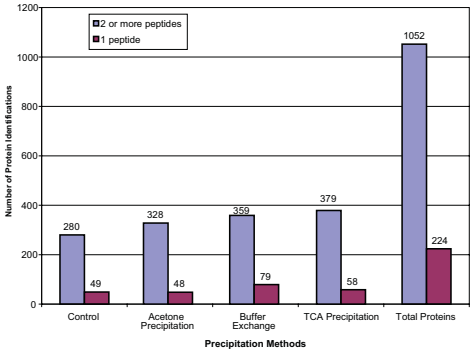
LC-MS/MS Identification

Samples were dissolved in 0.1% TFA and 5 µL was injected onto a Discovery C18 reverse phase HPLC column (15 cm × 2.1 mm, 5 µm particle size). This separation system made use of water and acetonitrile, both of which contained formic acid. Peptides were detected and identified using a linear ion trap mass spectrometer (Thermo LTQ) set to perform tandem MS on the top ten ions in each spectrum. An exclusion list was used to prevent excessive interrogation of any one peptide. Datasets were searched against the UniProt human database using the SEQUEST algorithm.

Depleted Plasma



Effect of Protein Precipitation Methods on LC MS/MS Protein Identifications



Methods Generating Each Identified Protein from Two or More Peptides

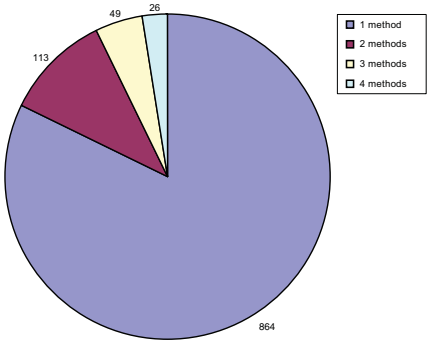
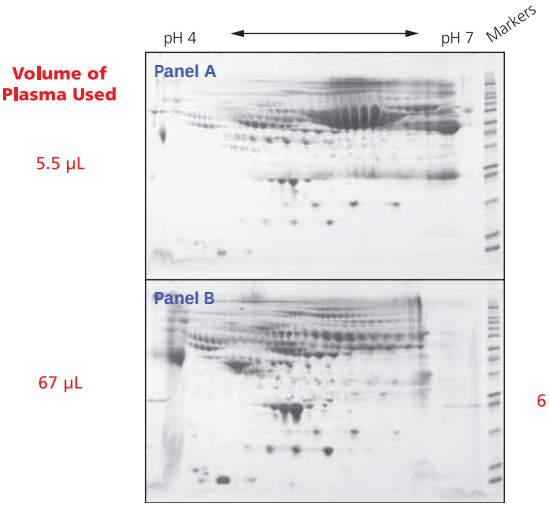


Figure 1. Sample Prep Methods Significantly Affect Protein Identifications

- More proteins (17–35%) were identified following the use of one of the 3 sample preparation methods than in the depleted control sample.
- 82% of the 1052 total proteins identified with 2 or more peptides were found in only one sample.
- Only 18% of the identified proteins were observed in more than one sample.
- Duplicate injections for each sample revealed 96–98% correlation of identified proteins.

A human plasma sample was depleted of 20 high abundance proteins. Depleted plasma (230 µg) from 90 µL of original plasma was further processed by either buffer exchange, acetone precipitation, or TCA precipitation. The samples were trypsin-digested, dried, and redisolved with 50 µL of 0.1% TFA. Duplicate 5-µL injections of each were introduced into a LC-MS/MS instrument (LTQ, Thermo). The LC separations were 200 min. Protein identifications were made based on a 99% probability. The protein identifications from each of the sample preparation methodologies were merged from duplicate injections. The data sets were separated by protein identifications made from either one peptide or 2 or more peptides. A table of all protein identifications from all 4 samples was created which included the XC Score and the number of peptides for each identification. This data set is available separately due to its size.

Unmasking and Increased Loading Capacity



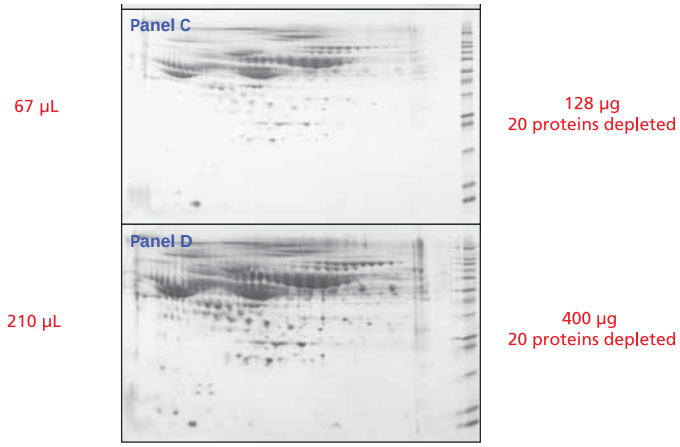


Figure 2: Depletion of 20 proteins allows for higher loads and visualization of low abundance proteins.

- 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 D vs. Panel B).
- Depletion of 20 proteins allows for a 38-fold increase in the load of low abundance proteins compared with whole plasma (undepleted) (Panel D vs. Panel A).

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. 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.

Bound Proteins

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	IGHA1_HUMAN (P01876) Ig alpha-1 chain C region	70	7	21
5	IGHA2_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	IGHD			
19	TTHY_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_METJA (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
KIRR3_HUMAN (Q8IZU9) 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 (Q96FI4) 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 3: 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 the Methods section. A sample was injected onto a C18 column and separated with a 200-minute acetonitrile gradient and the peptides identified via LC-MS/MS (LTQ, Thermo). Protein identifications were made based on a 99.9% probability.

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.
- 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.
- Non-specific binding is extremely low.
- Multiple sample preparation methods will increase the number of successful protein identifications.

References

1. Anderson, N. L. and Anderson, N. G. The Human Plasma Proteome. *Mol. Cell. Proteomics* **2002**, *1*, 845.
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