Human Serum and Plasma Protein Depletion: Novel High Capacity Affinity Column for the Removal of the ‘Top 14’ Abundant Proteins

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Introduction

Serum and plasma represent the most complex sample of the human proteome, composed of the homeostatic blood proteins as well as tissue leakage proteins. The tremendous complexity of this biofluids proteome presents extreme analytical challenges in comprehensive analysis due to the wide dynamic range of protein concentrations (spanning over 10 orders of magnitude). Consequently, robust sample preparation methods remain one of the important steps in the proteome characterization workflow.

Depletion of high-abundant proteins in serum and plasma has become an essential, routine and accepted technique. These high-abundant protein components interfere with identification and characterization of important low-abundant proteins by limiting the dynamic range for mass spectral and electrophoretic analyses. We are presenting the results on a new column for the specific depletion of 14 high-abundant proteins from serum and plasma. Through depletion of the 14 high-abundant proteins we are removing ~94% of the total protein mass. The depletion process is robust, easily automated and highly efficient (30 min.). The column depletes the 14 targeted proteins reproducibly during 200 runs and has excellent depletion efficiency as determined by ELISA. Results on the identification of the bound proteins indicate specific removal of the targeted proteins.

We have depleted plasma of 14 high-abundant proteins and performed a subsequent fractionation using a high-recovery macroporous reversed-phase (mRP) C18 column under optimized reversed phase conditions. The chromatographic conditions and methods enabled high protein recovery while permitting robust and reproducible fractionation. The collected column fractions were trypsin-digested and analyzed on a microfluidic HPLC-Chip/MS system, providing a reliable and fast peptide separation combined with ease of use, robust ionization and fast data acquisition.

High-abundant protein depletion and RP fractionation of plasma showed an improved dynamic range for proteomic analysis resulting in reduced ion suppression in electrospray MS. The multi-dimensional workflow approach presented here allowed the identification of low-abundant plasma proteins.

Experimental

Affinity Column

The newly developed column is an extension and improvement on the Agilent Multiple Affinity Removal approach described and evaluated previously (1-3). This material has a novel attachment process for affinity binders, including, and improved from, the affinity-selected polyclonal IgGs that have previously been employed. The column requires a two buffer system for operation. Buffers A and B are optimized to minimize co-adsorption of non-targeted proteins and to ensure reproducibility of column performance and long column lifetime. Buffer A – a salt-containing neutral buffer, pH-7.4, used for loading, washing and re-equilibrating the column and Buffer B – a low pH urea buffer used for eluting the bound high-abundant proteins from the column.

Sample preparation

Before injection onto a high capacity multiple affinity column, citrated human plasma (Rockland – D519-04) was diluted 4X with Buffer A. The sample was transferred to a 0.22 µm spin tube and centrifuged for 1 min. at 16,000 x g to remove particulates. Diluted plasma was kept at 4°C.

ELISA analysis of the flow-through fraction

Standard sandwich enzyme-linked immunosorbent assays (ELISA) were used to determine the completeness of removal of targeted proteins from human plasma. Briefly, assay plates were coated with 100 µl of flow-through fraction proteins diluted 1:10 in Buffer A. After an overnight incubation at 4° C, plates were washed with PBS and the nonspecific binding sites were blocked with 200 µl of blocker solution (Bio-Rad) for 2 hours.
After washing plates with PBS, 100 µl of anti-human antigen antibodies were added in blocker solution. Plates were incubated for 2 hours, washed, a secondary HRP-conjugated antibody was added for 1 hour. After washing with PBS, liquid substrate (TMB) was added and the absorbance was measured at 655 nm.

SDS-PAGE

Flow-through proteins and bound proteins were buffer exchanged into PBS and concentrated. Protein concentrations were analyzed using a BCA protein assay kit (Pierce). Samples were stored at –70°C until analysis. SDS-PAGE analysis was carried out using Invitrogen Tris-Glycine pre-cast gels (4-20% acrylamide, 10 wells, 1mm). Proteins were visualized by Coomassie Blue staining with GelCode Blue (Pierce).

mRP Protein Fractionation

For the direct analysis of the flow-through fraction, the sample was simultaneously desalted and fractionated with a macroporous Reversed Phase (mRP-C18) protein separation column (Agilent) according to Martosella J. et.al (4). The 32 fractions were taken to complete dryness with a Speed-Vac.

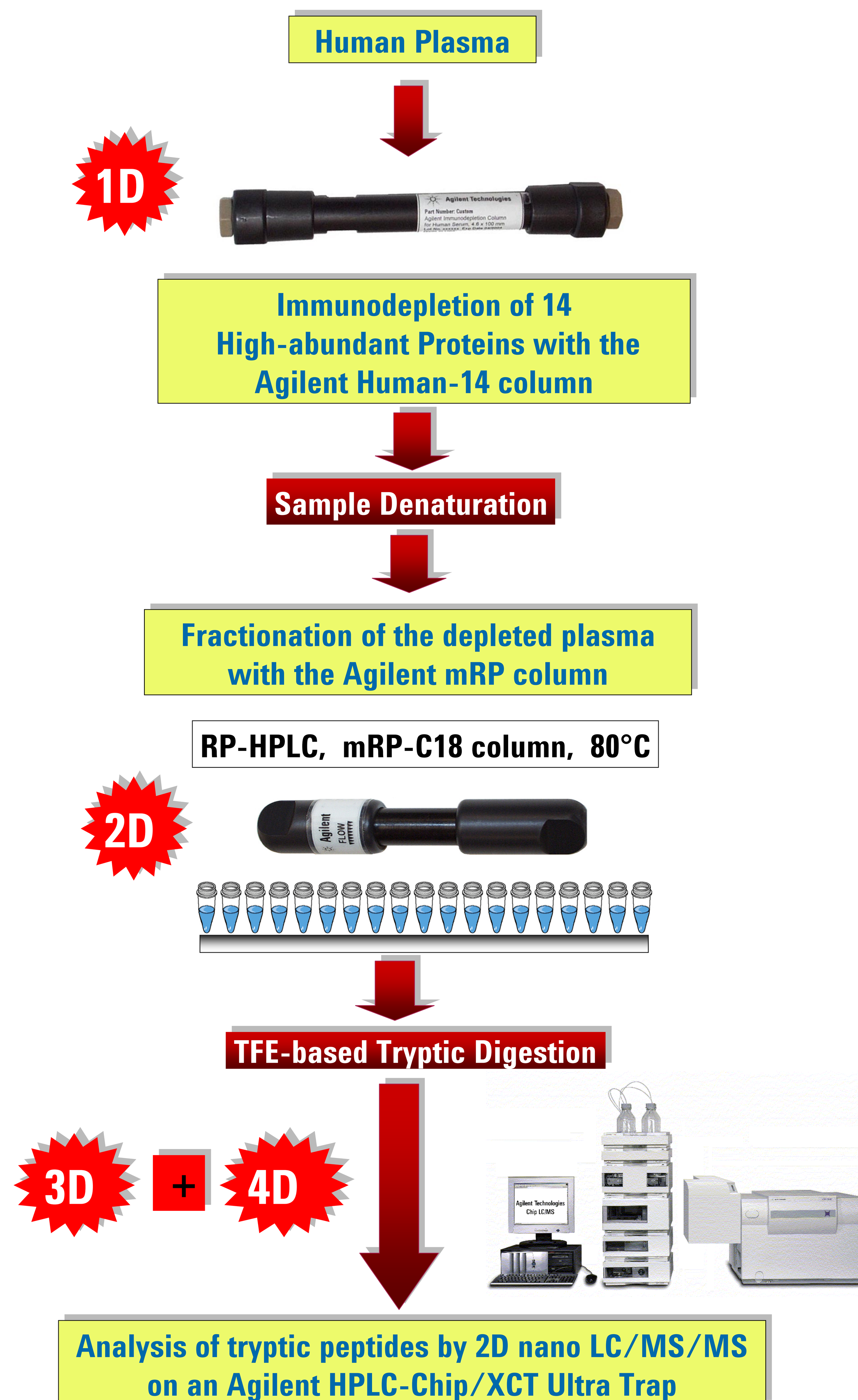
LC/MS/MS Analysis

To analyze the specificity of the immunodepletion, the bound fraction was resolved by SDS-PAGE. The entire bound fraction lane was cut into bands and processed with "In-gel trypsin digestion kit" (Agilent Technologies). Peptides were analyzed by LC/MS/MS on an Agilent HPLC-Chip / XCT Ultra Trap. Results were processed by Spectrum Mill software (Agilent Technologies).

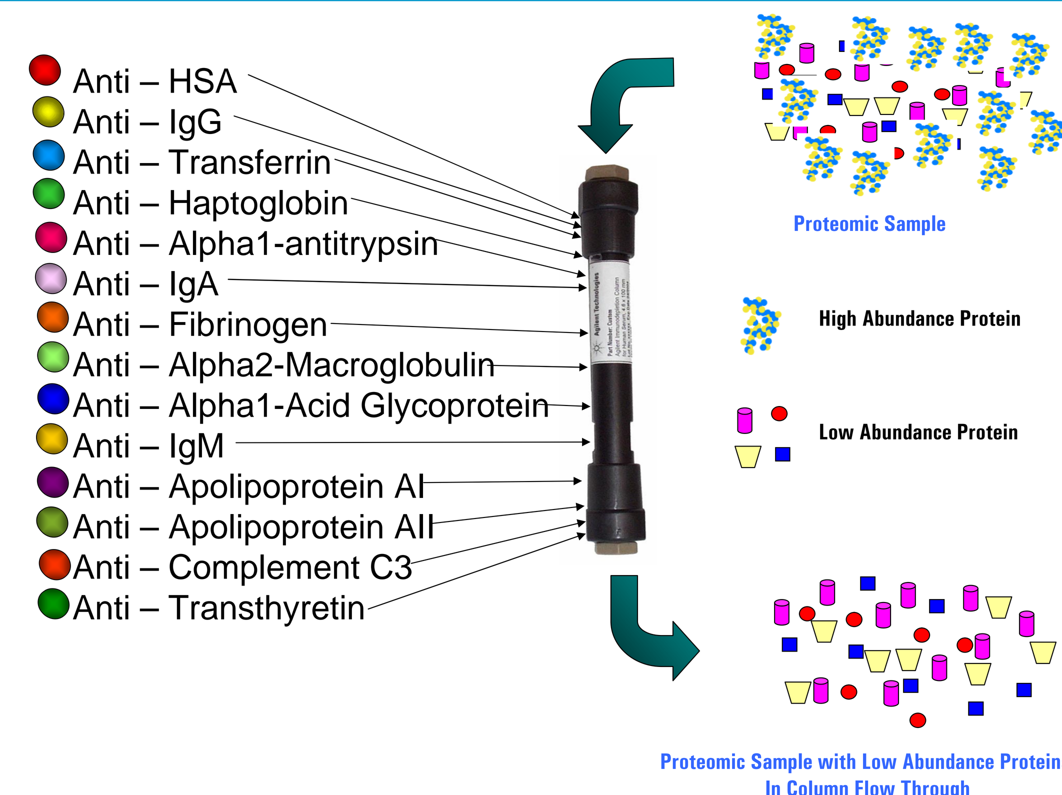
The dried fractions from the mRP column were digested using a Trifluoroethanol-based protocol (5). Peptides were analyzed by 2D nano LC/MS/MS on an Agilent HPLC-Chip / XCT Ultra Trap.

- Salt Steps: sample (0), 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000 mM sodium chloride.
- RP Mobile Phase: [A]-H_2O w\0.1% FA, 3% ACN & [B]-ACN w\ 0.1% FA, 3% H_2O – 500 nL/min
- Enrichment Mobile Phase: H_2O w\0.1% FA, 3% ACN – 5 µL/min
- RP Gradient: 0% B @ 4 min., 8% B @ 4.25 min., 35% B @ 39 min., 50% B @ 44 min., 95% B @ 44.1 min., 95% B @ 46 min., 0% B @ 47 min.

Experimental Workflow

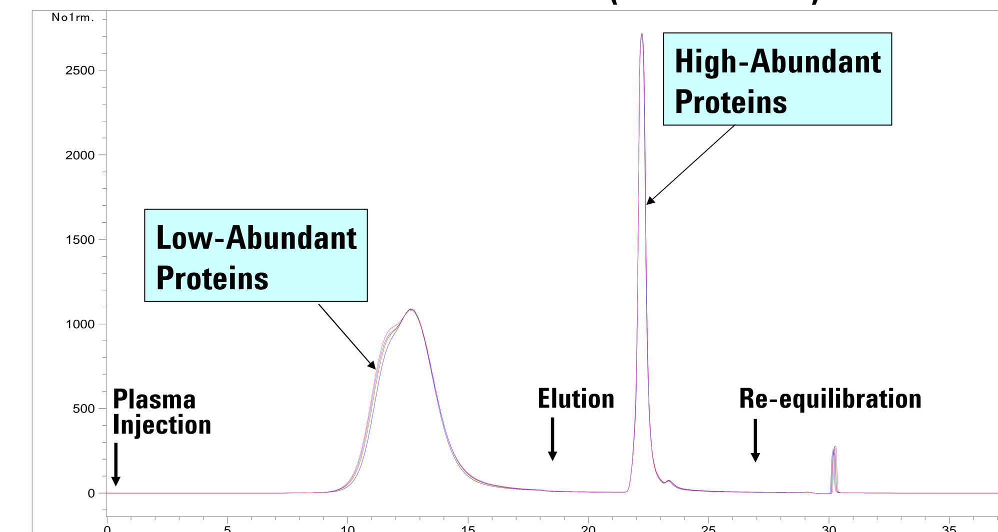


Human-14 Column

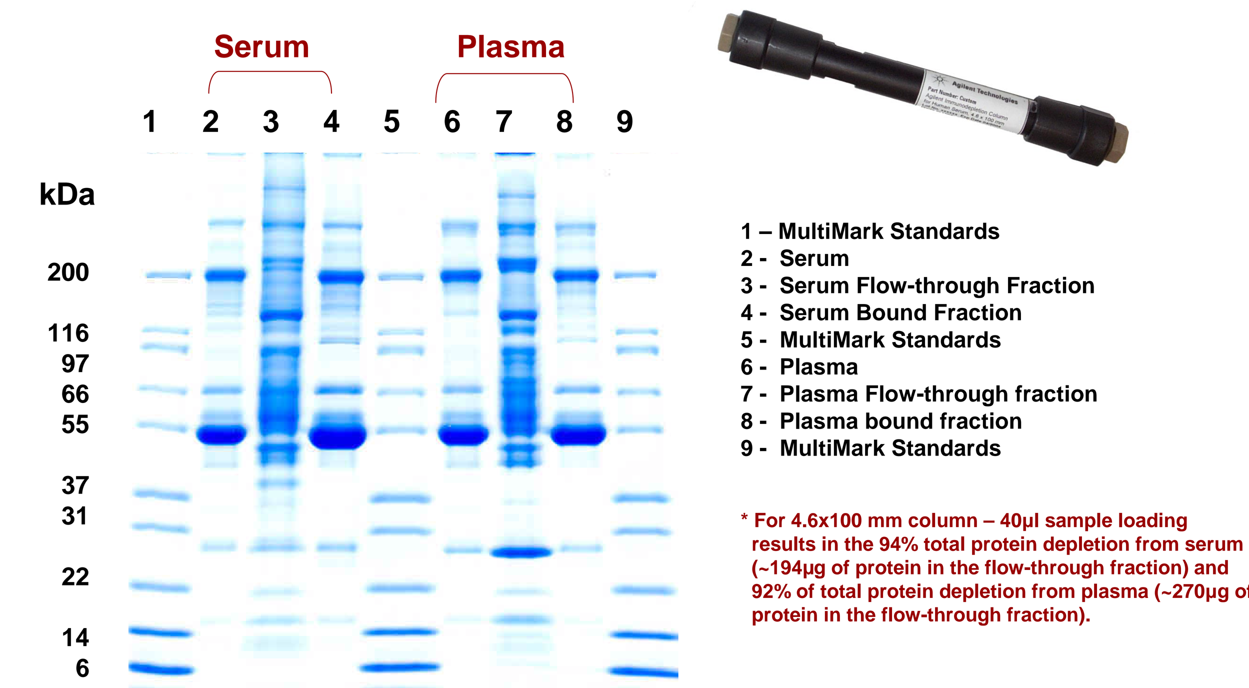


Column chromatograms during 200 runs

Overlay of chromatograms from run 1, 100, 150, and 200 on a Human-14 column (4.6x100 mm)



SDS-PAGE analysis of fractions from Human-14 column



* For 4.6x100 mm column – 40µl sample loading results in the 94% total protein depletion from serum (~194µg of protein in the flow-through fraction) and 92% of total protein depletion from plasma (~270µg of protein in the flow-through fraction).

Bound Fraction Analysis

Proteins identified in the serum bound fraction by LC/MS/MS

1	Human Serum Albumin
2	Immunoglobulin G
3	Immunoglobulin M
4	Immunoglobulin A
5	Haptoglobin
6	Transferrin
7	Alpha1-Anti-trypsin
8	Alpha2-Macroglobulin
9	Complement C3
10	Alpha1-Acid Glycoprotein
11	Apolipoprotein AI
12	Transthyretin (prealbumin)
13	Apolipoprotein AII
14*	Apolipoprotein B-100
15*	Plasma protease C1 inhibitor
16*	Zinc-alpha-2-glycoprotein
17*	Apolipoprotein L1

* Untargeted proteins not quantitatively removed

The serum bound fraction lane was cut into 22 bands and proteins were trypsin-digested and eluted from the gel. Peptides were analyzed by LC/MS/MS on an Agilent HPLC-Chip / XCT Ultra Trap as described in the experimental section.

The lists of proteins for each band were combined and are shown to the left.

References

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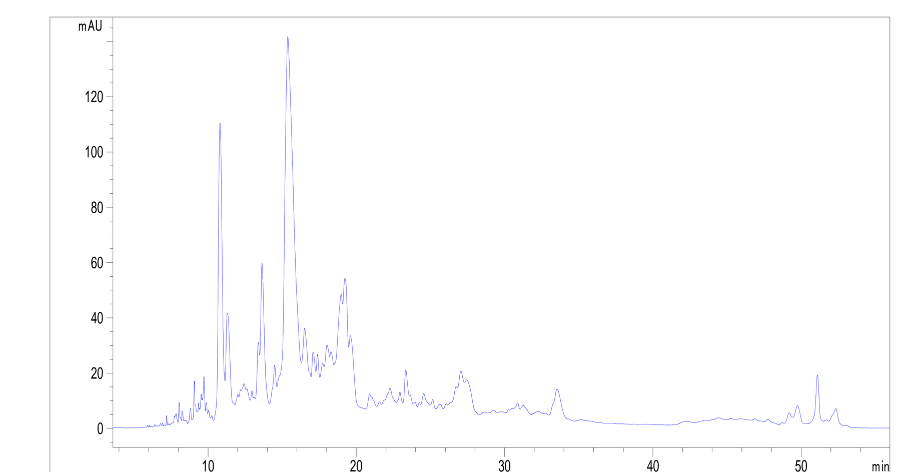
ELISA results for the depletion efficiency after 200 runs

Sample	MAC	AGP	IgM	ApoAI	ApoAII	C3	Transthyr.	Fibr.
Serum, 40ul	99.60%	99.60%	99.30%	99.20%	96.00%	96.00%	99.30%	
Plasma, 40ul	99.30%	99.50%	99.50%	98.40%	95.00%	98.60%	97.50%	97.6%

- Level I targeted proteins - HSA, Transferrin, IgA, IgG, Haptoglobin and Anti- trypsin were depleted with the efficiency of 99% and higher after 200 runs.

RP Fractionation of Depleted Plasma



- The mRP column with optimized reversed phase conditions provides >98% of protein recovery (4).

4D LC/MS analysis of flow-through fractions

Partial representation of the low abundant proteins found

Number	Protein Name	Accession #	Distinct Peptide #
1	Insulin-like growth factor binding protein 3 (ng/mL (6))	P17936	6
2	Transitional endoplasmic reticulum ATPase (TER ATPase)	P55072	7
3	Intercellular adhesion molecule-2 precursor (ICAM-2)	P13598	4
4	Mitogen-activated protein kinase 3 (MAPK)	Q8IVH8	6
5	Polycystic kidney and hepatic disease 1 precursor	Q8TCZ9	5
6	Dopamine beta-monooxygenase precursor	P09172	3
7	ADAM 10 precursor	O14672	4
8	Integrin alpha-1 (CD49a)	P56199	4
9	Peripheral-type benzodiazepine receptor-associated protein 1 (PRAX-1)	O95153	4
10	Insulin-like growth factor binding protein 4 precursor (IGFBP-4)	P22692	2
11	6-phosphofructokinase, liver type	P17858	4
12	cGMP-dependent protein kinase 1, beta isozyme	P14619	3
13	Cadherin-13 precursor (ng/mL)	P55290	3
14	Serine/threonine-protein kinase MAK	P20794	3
15	Insulin-like growth factor binding protein 6 precursor (IGFBP-6)	P24592	2
16	Tyrosine-protein kinase JAK1	P23458	3
17	Aminopeptidase N	P15144	2
18	Tumor necrosis factor, alpha-induced protein 2 (pg/mL)	Q03169	2
19	Protein kinase C, mu type (nPKC-mu)	Q15139	2
20	Interleukin-1 receptor-like 1 precursor (pg/mL)	Q01638	2
21	Cyclin T2	O60583	2
22	Interleukin-27 beta chain precursor (IL-27B) (pg/mL)	Q14213	2
23	LIM domain kinase 1 (LIMK-1)	P53667	3
24	Protein-tyrosine phosphatase precursor	Q12913	2

Conclusion

-Human-14 Multiple Affinity Removal Column depletes the top 14 abundant proteins from human serum, plasma, CSF, and other biological fluids. Column is extremely robust and performs reproducibly for over 200 runs.

-Depleted plasma (denatured) was directly injected on the mRP-C18 column. Each step of the presented multidimensional proteomic analysis was optimized to allow a smooth workflow with minimum sample manipulation (no sample dialysis, buffer exchange or precipitation).

-The combination of immunoaffinity chromatography with high recovery RP-HPLC fractionation and subsequent peptide analysis on HPLC-Chip/MS expanded the dynamic range of proteomic analysis, and allowed the identification of low-abundant proteins (ng/ml-pg/ml range).

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