Evaluation of Methods for Removal of Highly Abundant Human Plasma Proteins

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Overview

Three new commercially available antibody-based depletion approaches have been compared to the earlier Agilent 7-antibody method for removal of high-abundance proteins in human plasma. The results showed good depletion efficiency and reproducibility for all three new methods investigated. Signals from low-abundance plasma proteins were significantly enriched with the extended level of antibody removal, especially for Genway two-antibody column, two–step depletion approach.

Introduction

Plasma protein concentrations have extremely wide dynamic range. The top 12 to 14 proteins make up ~ 96% of plasma by mass. The sensitivity for plasma proteomic analysis and biomarker discovery is often limited by interferences and charge suppression from high-abundance proteins. One way to overcome this dynamic range issue is to use affinity columns to specifically remove sets of high-abundance proteins. Agilent and GenWay Biotech/Beckman Coulter are lead companies developing commercial products for this application.

Agilent Technologies offers an antibody column designed to remove 7 proteins from human plasma in a single step. This column has been used in our biomarker discovery platform routinely and has proven to be beneficial and reliable. More recently, Agilent has expanded its antibody panel to include 14 antibodies. GenWay Biotec offers not only the one-step but also two-step removal products. The first IgY-12 or IgY-14 column removes 12 or 14 abundant proteins, mostly in common to the Agilent column. Then, a second antibody "SuperMix" column with antibodies produced against the flow-through proteins of IgY-12, is applied to the flow-through of the first IgY-12 or IgY-14 column, resulting in a further depletion. Here we present comparative evaluation of these depletion methods for detecting low-abundance human plasma proteins.

Table 1: Summary of the evaluated depletion methods

	One-step depletion			Two-step depletion	
Protein Name	**Agilent Hu-7 removal**	**Agilent Hu-14 removal**	**GenWay IgY-14 removal**	**GenWay /Beckman IgY-12 removal**	**GenWay IgY-12 & SuperMix removal**
Albumin	*	*	*	*	*
IgG Total	*	*	*	*	*
Transferrin	*	*	*	*	*
Fibrinogen	*	*	*	*	*
IgA Total	*	*	*	*	*
Haptoglobin	*	*	*	*	*
Alpha-1-antitrypsin	*	*	*	*	*
A2 Macroglobulin		*	*	*	*
IgM Total		*	*	*	*
Apo A-I		*	*	*	*
Apo A-II		*	*	*	*
Alpha-1-acid glycoprotein		*	*	*	*
Complement component 3		*	*		*
Apolipoprotein B			*		
Transthyretin (prealbumin)		*			*
Examples of "SuperMix" depletion results					
Complement Factor H					*
Complement component 4B					*
Complement factor B					*
Plasma retinol-binding protein					*
Ceruloplasmin (Ferroxidase)					*
kininogen					*
Alpha-1-microglobulin (AMBP protein)					*
Inter-alpha (globulin) inhibitor H4					*
Plasminogen precursor					*

* removed protein

Methods

Experiment #1. For comparison of one-step removal methods, high-abundance proteins were depleted from a pooled EDTA human plasma sample (5 healthy donors from the Stanford blood bank) with a) the 7-antibody column (Agilent, Hu-7), b) the 14-antibody column (Agilent, Hu-14, prototype), and c) another 14-antibody column (GenWay, IgY-14).

Experiment #2. For comparison of the two-step removal method, three fractionated samples were purchased from GenWay Biotec, also prepared from an EDTA human plasma control sample. In this experiment, the 12-antibody column (Beckman/GenWay, IgY-12) was used for the first step of protein removal. The elution from the IgY-12 column bound protein represents the first sample investigated. For the second protein removal step, a second antibody column (GenWay, SuperMix) was used to further deplete the already IgY-12 depleted sample, with the elution of the "SuperMix" bound and the flow-through material representing the second and third samples investigated, respectively.

A small fraction of each sample investigated was used to determine total protein amount using BCA assay and the rest was concentrated, reduced, alkylated and digested by trypsin prior to a one-dimensional on-line capillary C-18 LC-ESI-MS or LC-ESI-MS/MS analysis. Approximately 10 µg fractionated plasma proteins were injected into the mass spectrometer.

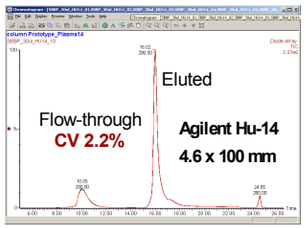
Label free MS profiling and MS/MS peptide/protein identification: Relative fractionation and depletion efficiencies were evaluated based on the MS signal of LC-ESI-TOF-MS (Waters, LCT) runs of the depleted samples. Peptide identification data were acquired on a LC-ESI-MS/MS system with a linear ion trap (ThermoFisher, LTQ). Each sample investigated was subjected to 4 separate MS/MS library runs;1) m/z range 300-700, ions 1-10; 2) m/z range 700-2000, ions 1-10; 3) m/z 300-700 range, ions 11-20; and 4) m/z 700-2000, ions 11-20. HPLC gradient: 0 to 40% ACN with 0.1% formic in 46 min. Proteins/peptides were identified using database searching with Mascot software against a human database containing reverse sequences. The Mascot score cutoff for identification at individual peptide level was determined based reaching a local 30% false discovery rate using reverse sequence "hits." A typical cutoff score is 38.

Results

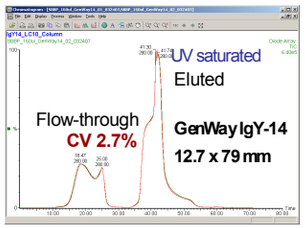
Experiment #1: Comparison of one-step removal methods

Reproducibility

Figure 1: Overlay of UV traces @280 nm of antibody depletion of multiple injections with two difference size of new 14-antibody columns.



Plasma sample Injected (uL)	% protein removal by BCA
20	92.8%
30	91.6%
40	91.4%
50	91.3%



Plasma sample Injected (uL)	% protein removal by BCA
140	96.3%
160	96.2%
180	96.0%
200	96.2%

Depletion efficiencies for high-abundance proteins

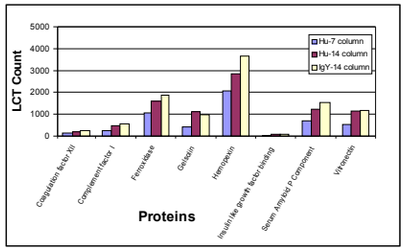
Table 2: Comparison of one-step antibody removal efficiency for depleted proteins

Protein Name	**Agilent Hu-7 removal**	**Agilent Hu-14 removal**	**GenWay IgY-14 removal**	**Hu-7 LCT count**	**Hu-14 LCT count**	**IgY-14 LCT count**
% of total protein amount removed by BCA	**88%**	**92%**	**96%**			
Reproducibility (CV of flow-through @280 nm)	**0.7%**	**2.2%**	**2.7%**			
Albumin	*	*	*	n/a	n/a	n/a
A1-antitrypsin	*	*	*	52	10	n/a
Fibrinogen	*	*	*	28	6	11
Haptoglobin	*	*	*	159	79	95
Transferrin	*	*	*	n/a	48	n/a
A2 Macroglobulin		*	*	1670	103	29
A1acid glycoprotein		*	*	1840	9	n/a
Apo A-I		*	*	3590	705	4
Apo A-II		*	*	4180	1098	224
Complement C3		*	*	1210	50	10
Transthyretin		*		425	5	253
Apo-CIII			*	1400	370	9
Apo B			*	358	130	n/a

LCT count from Hu-14 or IgY-14 column was scaled to the loading of Hu-7 column with equivalent plasma 1.25 µL pre-depletion loaded on LCT.

Signal enrichment for low-abundance proteins

Figure 2: Comparison of signal enrichment for non-depleted proteins with one-step depletion methods



For Hu-14 and IgY-14 columns, data were obtained with the LCT and LTQ loading of sample equivalent to 5 µL of non-depleted plasma, whereas for Hu-7 column, equivalent 1.25 µL pre-depletion plasma was loaded for comparison.

Table 3: Comparison of one-step antibody depletion methods for non-depleted low-abundance protein identifications, each sample investigated was subjected to 4 separate MS/MS runs with different m/z ranges.

	Agilent Hu-7		**Agilent Hu-14**		**GenWay IgY-14**	
% of total protein amount for flow-through	**12%**		**8%**		**4%**	
Reproducibility (CV of flow-through@280 nm)	**0.7%**		**2.2%**		**2.7%**	
Protein Name	**LTQ #scans**	**# unique peptides**	**LTQ #scans**	**# unique peptides**	**LTQ #scans**	**# unique peptides**
Coagulation factor XII	5	5	7	7	6	6
Complement factor I	6	6	12	11	11	11
Gelsolin	10	10	27	21	20	19
Insulin like growth factor binding	2	2	7	7	8	8
Serum Amyloid P Component	6	5	8	8	6	5
Vitronectin	3	3	6	6	5	5
Complement component C6 precursor	5	5	8	8	17	16
Zinc-alpha-2-glycoprotein precursor	2	2	2	2	13	13
Complement C2 (C3/C5 convertase)	5	4	8	7	15	14
Coagulation factor X			10	9	17	15
L-selectin (Lymph node homing receptor)					2	2
Leucine-rich alpha-2-glycoprotein (LRG)			1	1	8	7
Hepatocyte growth factor-like protein	1	1	1	1	4	4
Extracellular matrix protein 1	3	3	5	5	5	5
Beta-2-microglobulin			1	1	4	3
Mannose-binding protein C					2	2

Since this experiment, Agilent has improved the loading protocol for its 14-antibody column, new results are not included.

Experiment #2: Comparison of two-step removal fractions

Table 4: Summary of protein/peptide identification from three fractions of the IgY-12 plus "SuperMix" depletion method, starting with 250 µL plasma. Each fraction was subjected to 4 separate MS/MS runs with different m/z ranges.

	GenWay /Beckman IgY-12 eluted fraction		**GenWay SuperMix eluted fraction**		**GenWay SuperMix flow throught fraction**	
% of protein amount by BCA	**89.0%**		**8%**		**3%**	
Reproducibility (CV of BCA from 4 replicates)	**1.0%**		**10.0%**		**18.0%**	
Protein Name	**LTQ #scans**	**# unique peptides**	**LTQ #scans**	**# unique peptides**	**LTQ #scans**	**# unique peptides**
Albumin	447	85	38	27		
Ig gamma-1 chain C region	66	25	2	2		
Transferrin	142	53	1	1		
Fibrinogen	17	12	7	7	3	2
Ig alpha-1 chain C region	13	8	2	2		
Haptoglobin	80	29	3	3		
Alpha-1-antitrypsin	46	21				
A2 Macroglobulin	108	50	6	6	113	36
Ig mu chain	19	14			1	1
Apo A-I	74	23				
Apo A-II	4	3				
Alpha-1-acid glycoprotein	20	11			1	1
Complement component 3	1	1	275	97	49	24
Complement Factor H	4	4	54	37	8	7
Alpha-2-HS-glycoprotein (Fetuin-A)	10	6	5	3	43	10
Complement component 4B			112	59		
Complement factor B			55	29	2	2
Plasma retinol-binding protein			6	3		
Ceruloplasmin (Ferroxidase)			81	43	11	7
Alpha-1-antichymotrypsin	1	1	10	7	123	13
vitamin D-binding protein			17	9	122	25
Apolipoprotein B	3	2			309	121
Transthyretin (prealbumin)	7	4			5	3
Prothrombin (Coagulation factor II)			3	3	72	25
Serum Amyloid P Component			6	4	1	1
Vitronectin			4	3	7	4
Complement component C6 precursor			4	3	11	10
Coagulation factor XII					12	6
Insulin like growth factor binding protein					20	12
Complement C2 (C3/C5 convertase)					58	20
Coagulation factor X					21	8
Hepatocyte growth factor-like protein					20	11
L-selectin (Lymph node homing receptor)					1	1
Leucine-rich alpha-2-glycoprotein (LRG)					1	1
Extracellular matrix protein 1					11	8
Cartilage oligomeric matrix protein					5	5
Mannose-binding protein C					3	2
Fibrillin-1					1	1
Galectin-3-binding protein					2	2
Beta-2-microglobulin					2	1

Conclusion

Several new antibody depletion methods have been compared with the earlier available Agilent 7-antibody removal method using pooled normal human plasma samples. The GenWay technology with two antibody column depletion (IgY-12 plus "SuperMix") results in detection of human plasma proteins at lower concentration than the either of the Agilent or GenWay one-step 14-antibody methods. For example, several lower abundance proteins, such as insulin-like growth factor-binding proteins, L-selectin, fibrillin-1, galectin-3-binding protein, cartilage oligomeric matrix protein, and mannose-binding protein C were enriched significantly and detected via a one-dimensional LC-MS/MS analysis. However, the detection of low abundance proteins using the "SuperMix" depletion strategy could come at the expense of not monitoring some intermediate or higher abundance proteins such as orosmucoid and HDL which can serve as biomarkers in certain disease/drug situations. Thus if resources permit, more than one method or one fraction will result in a more complete coverage. One possible scenario for this complementary approach is to use a one-dimensional chromatographic approach for the first column's depleted (eluted) material, and a one- or two-dimensional chromatographic approach for the "SuperMix" flow-through fraction.

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