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IgY14 and SuperMix immunoaffinity separations coupled with liquid chromatography-mass spectrometry for human plasma proteomics biomarker discovery

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Abstract

Interest in the application of advanced proteomics technologies to human blood plasma- or serum-based clinical samples for the purpose of discovering disease biomarkers continues to grow; however, the enormous dynamic range of protein concentrations in these types of samples (often >10 orders of magnitude) represents a significant analytical challenge, particularly for detecting low-abundance candidate biomarkers. In response, immunoaffinity separation methods for depleting multiple high- and moderate-abundance proteins have become key tools for enriching low-abundance proteins and enhancing detection of these proteins in plasma proteomics. Herein, we describe IgY14 and tandem IgY14-SuperMix separation methods for removing 14 high-abundance and up to 60 moderate-abundance proteins, respectively, from human blood plasma and highlight their utility when combined with liquid chromatography-tandem mass spectrometry for interrogating the human plasma proteome.

Keywords

SuperMix; IgY14; immunoaffinity; plasma proteomics; Biomarker discovery

1. Introduction

Human bodily fluids, especially blood plasma and serum, that contain signature proteins relevant to most human diseases are widely utilized sample types for biomarker discovery [1, 2]. However, the ability to detect and identify specific is often hampered by the “masking” effect caused by high-abundance proteins (HAP) that dominate these biofluids. For example, the 22 most abundant proteins in the human plasma proteome (in which protein concentrations span a dynamic range >10 orders of magnitude [3]) account for 99% of the total protein mass. Detection of candidate biomarkers present at ng/mL to pg/mL levels against a background of HAP that include albumin present at mg/mL levels is a formidable analytical challenge for current proteomics technologies. As a result, it is often necessary to separate or remove HAP and moderate-abundance proteins (MAP) from

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plasma/serum to enhance the detection of low-abundance proteins (LAP) and improve proteome coverage.

Immunoaffinity separations using immobilized antibodies have become the most commonly utilized strategies for removing HAP in blood plasma and serum. Affinity-purified polyclonal antibodies that are typically immobilized onto either chromatographic matrices or microbeads by cross-linking are used as immunoaffinity reagents to specifically remove target proteins [4–6]. Multiple HAP can be removed by optimizing a mixture of different antibody-immobilized beads within the partitioning column, as initially demonstrated by Piper *et al.* [4].

Many immunoaffinity products are now commercially available for simultaneous removal of multiple HAP from human plasma or serum. Most of these products are designed for single-stage separations that remove up to 20 HAP, depending upon the product. A multiple affinity removal system (MARS) from Agilent Technologies was one of the first immunoaffinity depletion systems to be commercialized. Initially, this product consisted of a mixture of polyclonal IgG antibodies to six HAP (serum albumin, IgG, IgA, transferrin, α -1-antitrypsin, and haptoglobin) attached to polymeric beads. Antibodies attached to the polymeric support through their Fc regions provide easy protein access to the affinity binding sites, and reported depletion efficiencies are >99% [7]. Later additions to the product line included a MARS-7 column that targets the original six proteins plus fibrinogen [8–10] and a MARS Hu-14 column that removes α ₁-acid glycoprotein, α ₂-macroglobulin, IgM, apolipoproteins A-I & A-II, complement C3, and pre-albumin in addition to the original six proteins and fibrinogen [10, 11]. Sigma-Aldrich offers the ProteoPrep[®] 20, which uses a mixture of polyclonal IgGs and single-chain antibodies to remove 20 HAP in human plasma/serum [12, 13].

A family of avian polyclonal immunoglobulin yolk (IgY) antibodies-based immunoaffinity products includes the Seppro[®] IgY developed by GenWay Biotech. The Seppro[®] IgY products (IgY12 and IgY14) consist of individual anti-HAP IgY beads blended to form mixtures that specifically remove either 12 or 14 HAP in human plasma with high reproducibility, as well as low-level binding of non-target proteins [5, 14–16]. As immunoaffinity reagents, the IgY products have several advantages over IgG-based immunodepletion systems, including high affinity for HAP; less cross-reactivity to non-target proteins, which makes IgY antibodies more target-specific; target proteins are readily stripped from their cognate IgYs, which allows the IgY beads to be recycled multiple times; application to other mammalian proteomes due to a broader range of anti-human IgYs [3, 5, 17–19].

While a number of single-stage immunoaffinity separation techniques have been demonstrated for removal of HAP [15], MAP remaining in the flow-through fraction still present a challenge for detection of LAP present at low ng/mL or even lower concentration levels. Recent application of a SuperMix column in tandem with an IgY12 column demonstrated removal of both HAP and MAP, effectively enriching LAP prior to proteomics analysis [6, 20]. A commercial IgY14 column is now available, which removes two additional abundant proteins (C3 and apolipoprotein B) (Fig. 1A). Note, all Seppro[®] IgY immunodepletion products are currently available from Sigma-Aldrich in both bulk and liquid chromatography (LC) column formats.

Herein, we present a brief overview of single-stage IgY14 and tandem IgY14-SuperMix immunoaffinity separations [6] as two of the most commonly applied strategies in proteomics-based biomarker discovery studies. Following the brief overview, we describe the detailed experimental protocols and highlight their utility in proteomics applications.

While our applications exemplify the use of immunoaffinity separations in combination with LC-tandem mass spectrometry (MS/MS), in principle, these separations can be coupled with any downstream proteomics technologies for biomarker discovery or candidate verification.

2. Overview of IgY14 and SuperMix strategies

All analytical detection technologies including LC-MS have a limited dynamic range of detection. The range of protein concentrations in the human blood plasma spans >10 orders of magnitude, which is far greater than the dynamic range of detection afforded by LC-MS technologies (typically 4–5 orders of magnitude). As a result, strategies are necessary to reduce the range of protein concentrations and enhance the ability to detect LAP.

The IgY14 column is designed to remove the 14 most abundant proteins in human plasma that constitute 90–95% of the total protein mass (Fig. 1A). Individual IgY antibodies immobilized on microbeads against these 14 proteins are mixed in optimal amounts based on relative protein abundances to generate the stationary phase mixture for packing IgY14 columns. After loading a sample onto the column, >99% of the 14 abundant proteins are retained and then can be eluted as the bound fraction. MAP and LAP in the ‘depleted’ flow-through fraction are enriched by 10-fold for the same sample volume as the original sample. In other words, the dynamic range of protein concentrations in the depleted fraction is reduced by at least 10-fold.

The use of a SuperMix system to remove MAP (Fig. 1B) further enriches LAP. The column for this system is generated by using an IgY14-depleted plasma sample as a mixture of antigens to produce polyclonal IgY antibodies from immunized chickens. The IgY antibodies against MAP are purified using an antigen affinity column, after which the purified IgY antibodies are immobilized and packed into a SuperMix column (Fig. 2). When applied after IgY14 depletion, the SuperMix column captures an additional 50 or more MAP. The flow-through fraction contains mostly LAP in ~1% of the total starting plasma protein mass (Fig. 1B and Fig. 2). Collectively, tandem IgY14 and SuperMix separations provide ~100-fold enrichment of LAP in the flow-through fraction and at least two orders magnitude reduction in the dynamic range of protein concentrations. Application of these methods to enhance detection of LAP in blood plasma has been demonstrated for both human and mouse samples [6, 20].

3. Experimental methods

3.1. Human blood plasma sample

Human plasma protein concentration is determined by BCA protein assay (Pierce), after which samples are diluted 5-fold with Tris Buffered Saline (TBS) that contains 10 mM Tris-HCl and 150 mM NaCl, pH 7.4. Samples may contain particulate materials that can be removed by using a 0.45 μ m spin filter and centrifuging for 1 min at 9000 $\times$ g. Unless otherwise noted, all protein sample processing is performed at 4 °C.

3.2. Generation of IgY14 and SuperMix LC columns

IgY antibodies (Seppro® IgY products), which were originally developed by GenWay Biotech, are now available from Sigma-Aldrich. To make these antibodies, purified human plasma proteins (> 96% purity) are introduced into laying hens as antigens and affinity ligands. The resulting antibodies are isolated from the egg yolks that provide a high-yielding reservoir of easy-to-access antibodies [5, 18]. The antibodies are purified through affinity columns prepared by coupling antigens to CNBr-activated Sepharose™ 4B (GE Healthcare), after which the affinity-purified IgY antibodies are conjugated to UltraLink Hydrazide Gel (Pierce). In the conjugation process, 2–5 mg/mL IgY antibodies in phosphate buffered saline

(PBS) are oxidized with sodium periodate (NaIO_4) for 30 min at room temperature. Once dialyzed against PBS to remove residual oxidant, the antibodies are coupled to UltraLink Hydrazide Gel microbeads at 4 °C overnight. IgY-coupled microbeads are then thoroughly washed with 1 M NaCl, followed by TBS, and stored as a 50% slurry in TBS with 0.01% NaN_3 at 4 °C. The IgY immunodepletion column is prepared by blending individual anti-HAP IgY beads in optimal ratios based on the relative protein abundances of the 14 HAP in human plasma/serum.

The generation of a SuperMix column is similar to that of the IgY14 column. HAP in a plasma sample are initially depleted using an IgY14 affinity column, and the flow-through fraction (containing MAP and LAP) is then used to immunize chickens to generate a mixture of polyclonal IgY antibodies. As the IgY14-depleted flow-through fraction contains affinity ligands, the flow-through also is used to prepare an antigen affinity column for purifying antibodies from IgYs isolated from immunized chickens. Once purified, the mixture of IgY antibodies is conjugated to UltraLink Hydrazide Gel beads that are packed into an immunoaffinity column referred to as the SuperMix column (Fig. 2).

3.3. IgY14 and SuperMix immunoaffinity chromatography

3.3.1. Preparation of the LC system—IgY14 and SuperMix separations are performed using an Agilent 1100 series or similar type of LC system [15]. Most applications employ the IgY14 LC10 (10 mL bed volume) and the SuperMix LC5 (5 mL bed volume) columns. Overall protocols for using these columns generally follow the manufacturer's (Sigma-Aldrich) instructions. The following three buffers diluted 10× from stock solutions are used as the mobile phases: 1) dilution buffer (TBS) – 10 ~ mM Tris-HCl, 150 mM NaCl, pH 7.4; 2) stripping buffer – 100 mM glycine, pH 2.5; and 3) neutralizing buffer – 100 mM Tris-HCl, pH 8.0. Before attaching the column to the LC systems it is important to purge lines with these three buffers and then run the dilution buffer at a flow rate of 2 mL/min to check the system backpressure. The operational pressure includes not only the pressure introduced by the column, but also the system backpressure from the instrument. Note, the pressure introduced by the column is usually < 4 bar. Following attachment of the immunoaffinity separation column to the LC system, equilibrate the column with the dilution buffer for 20 min at a flow-rate of 2 mL/min to obtain a flat baseline, all the while monitoring at 280 nm. Two blank runs are then performed by injecting 500 µL of dilution buffer.

3.3.2. IgY14 LC10 separations—While the IgY14 LC10 column has a capacity for up to 250 µL human plasma or serum, based on our experience it is better to load 200 µL or less to avoid over-loading. The IgY14 LC10 separation consists of five steps (i.e., sample loading, washing, eluting, neutralization, and re-equilibration). The total cycle time using an Agilent 1100 series HPLC system is ~60 min [6, 15]. Up to 930 µL diluted and filtered sample (containing up to 185 µL original plasma) is injected onto the IgY14 LC-10 column at a flow rate of 0.5 mL/min for 20 min, after which the column is washed with the dilution buffer at a flow rate of 2.0 mL/min for 5 min. The IgY14 flow-through fraction is collected and stored at –80 °C until analysis. Bound proteins are eluted from the column using the stripping buffer at a flow rate of 2.0 mL/min for 15 min and collected as the IgY14 bound fraction. The column is then neutralized using the neutralizing buffer at a flow rate of 2.0 mL/min for 10 min and re-equilibrated with the dilution buffer at 2.0 mL/min for 10 min. The re-equilibrated column is ready for the next injection or may be stored at 4 °C in the dilution buffer that contains 0.02% sodium azide.

Following the IgY14 separations, the flow-through and bound fractions can be concentrated using Amicon® Ultra-15 (5 kDa nominal molecular weight limit, Millipore, Billerica, MA)

concentrators followed by buffer exchange to 50 mM NH_4HCO_3 (pH 8.0). Protein concentrations can then be determined by BCA protein assay (Pierce).

3.3.3. SuperMix LC5 separations—The concentrated IgY14 flow-through fraction can be subjected further to SuperMix LC5 partitioning on the same HPLC system in an off-line mode [6]. The separation procedures on the SuperMix LC5 column are similar to those described above for the IgY14 LC10 separation [6, 15]. Both the flow-through and bound fractions from the SuperMix column are collected and concentrated with buffer exchange to 50 mM NH_4HCO_3 .

3.3.4. On-line tandem IgY14-SuperMix separations—By introducing a six-port control valve to couple the two columns, automated tandem IgY14 LC10 and SuperMix LC5 separations can be performed (Fig. 3) [20]. The IgY14 and SuperMix LC5 columns connected by the six-port valve (Agilent, Santa Clara, CA) allow the plasma sample to pass through both columns following injection. Positions 3 and 5 on the valve are plugged to prohibit back-flush towards the waste or columns during valve switching. A tee union allows the liquid stream from the IgY14 or IgY14-SuperMix column to be directed towards the UV detector and subsequently towards the fraction collector. This configuration allows collection of the SuperMix flow-through and bound portions, as well as IgY14 bound portion in different fractions, and significantly improves the overall throughput and reproducibility of the tandem IgY-SuperMix immunodepletion systems [20].

Loading a diluted plasma or serum sample onto the tandem IgY14 and SuperMix separation system is performed using $1\times$ dilution buffer at a flow rate of 0.5 mL/min for 45 min to obtain the flow-through fractions from both columns (Fig. 3A). Afterwards, the IgY14 bound proteins are eluted by passing the stripping buffer to only the IgY14 column at a flow rate of 2.0 mL/min for 15 min and collected as the IgY14 bound fraction (Fig. 3B). The stripping buffer is then passed through to both columns at a flow rate of 1.0 mL/min for 20 min to elute the SuperMix bound fraction (Fig. 3C). Both columns are subsequently neutralized by passing neutralization buffer through at 1 mL/min for 20 min, and re-equilibrated with dilution buffer at 1 mL/min for 20 min for a total cycle time of 120 min. All collected fractions are concentrated in Amicon Ultra-15 concentrators followed by buffer exchange to 50 mM NH_4HCO_3 at pH 8.0.

3.3 Downstream proteomics analysis

Protein samples in the collected bound and flow-through fractions are amenable to downstream processing and proteomics analyses with various technologies. In the case of LC-MS/MS proteomics profiling, protein samples are typically subjected to trypsin digestion, and the resulting peptide samples are analyzed by LC-MS/MS, using ion-trap mass spectrometers as previously described [6]. In 2D-LC-MS/MS, strong cation exchange chromatography is applied for initial peptide fractionation prior to LC-MS/MS analyses. Peptides are identified by using SEQUEST or Mascot algorithms [6] to search MS/MS spectra against a pre-established human protein database. In our previous study, we employed static carboxamidomethylation of cysteine and dynamic oxidation of methionine criteria for the database search [6]. The false discovery rate of peptide identifications can be estimated by using an established decoy database searching methodology [21] and filtering criteria are applied to control the false discovery rate to be $<5\%$ at the unique peptide level.

4. Proteomics applications of IgY14 and SuperMix separations

In principle immunoaffinity separations can be coupled with a variety of different proteomics technologies to enable broad proteomics applications. We note that some of the data presented in the following applications were obtained using an IgY12 column, as at the

time of study, the IgY14 column had not yet been placed on the market [6]. Regardless, the results from the original study performed with the IgY12 column demonstrate the enhancement in LAP afforded by the SuperMix depletion, as well as overall reproducibility of the separation systems.

4.1. Enhanced coverage of LAP by SuperMix separations

A comprehensive list of proteins identified previously using 1D- and 2D-LC-MS/MS analyses [6] allows us to illustrate the enhanced LAP coverage achieved with the SuperMix separation. In a head-to-head comparison of the proteome coverage between IgY12 and SuperMix flow-through samples, ~64% and ~83% more proteins were identified in the SuperMix flow-through fraction in 1D- and 2D-LC-MS/MS analyses, respectively, than in than in the IgY12 flow-through fraction. Moreover, tandem IgY12-Supermix separations coupled with 2D-LC-MS/MS allowed identification of more LAP in human plasma with literature-reported concentrations below 100 ng/mL than did a single stage IGY12 separation, including identification of M-CSF and MMP8 that have ELISA-validated concentrations of 202 pg/mL and 12.4 ng/mL, respectively (See Fig. 4) [6]. These results clearly show that the use of tandem IgY12-SuperMix separations offers significant enhancements in detection of LAP and overall proteome coverage.

4.2. Reproducibility of IgY and tandem IgY-SuperMix separations

Both IgY12 [15] and tandem IgY12-SuperMix separations have proven reproducible [6], which is particularly important for quantitative applications in clinical proteomics. Good reproducibility was evident in immunoaffinity HPLC traces of tandem IgY14-SuperMix depletion for analyses of a quality control plasma sample obtained during early, middle, and late stage of the column life, respectively (Fig. 3D). Moreover, technical LC-MS/MS replicate analyses also demonstrated the reproducibility of the overall process based on the number of MS/MS spectra ("spectral count") that identify a given protein as shown in Fig. 5. Our experience over the past several years indicates that IgY12, IgY14 and SuperMix columns typically allow 100–150 reproducible separations within an approximate two-year shelf-life span.

Quality control analyses of a standard human plasma sample should be performed routinely to assess reproducibility of immunoaffinity separations over the column life time. The intensity ratios between three peaks (as shown in the chromatograms in Fig. 3D), as well as peak shapes and peak elution times, which should be reproducible over the column life-time, are used as the parameters for assessing column performance. We have typically observed rapid deterioration of peak shapes and elution times when > 100 separations are performed on a given column, an indicator that column performance was no longer acceptable.

4.3. Capture efficiency for HAP and MAP

The capture efficiency of the IgY12 and SuperMix columns for HAP and MAP can be estimated based on the observed spectral counts of target proteins from triplicate LC-MS/MS analyses of both bound and flow-through fractions. This estimation allows identification of target proteins that are efficiently captured by the column because either none or a minimum number of spectral counts should be observed in the flow-through fraction [6]. For example, we observed 45 MAP captured by the SuperMix column with >90% efficiency (Table 1), with ~35 additional proteins captured with 20–90% efficiency.

4.4. Perspective on high throughput applications

Both IgY14 and tandem IgY14-SuperMix immunodepletion strategies offer an effective way to dig deeper into complex biofluid proteomes for quantitative clinical applications. Unlike

other fractionation approaches that typically produce many fractions, the immunoaffinity separations only generate two (i.e., flow-through and bound) fractions with the added benefit of increased analytical throughput. Both single-stage IgY14 and tandem IgY14-SuperMix separations can be fully automated on an HPLC system to enable the processing of many samples [20, 22].

With a shelf-life of at least two years and capability for 100–150 separations per immunoaffinity column, column-to-column variations in a given large-scale study are minimized. We note that potential column-to-column variations have not been well studied to date and that such assessment would be necessary if a large-scale study involving several thousands of samples were to be performed using such technologies. However, given the observed overall good reproducibility of immunoaffinity separations, the IgY14 and IgY14-SuperMix strategies can be effectively coupled with downstream quantitative proteomics analyses for biomarker discovery, as well as for candidate biomarker verification. Different quantitative approaches such as label-free [23], spectral counting [24], O^{16}/O^{18} labeling [25, 26], iTRAQ labeling [27], and targeted quantification using LC-SRM-MS coupled with stable isotope dilution [8, 28] are all applicable and the choice of which to use would depend on the study design.

4.5. Potential pitfalls

While tandem IgY14-SuperMix immunoaffinity separations have been demonstrated effective for a range of applications, there are several potential pitfalls associated with the immunoaffinity methods that should be noted. First, a general concern for immunoaffinity strategies is the potential loss of target proteins caused by non-specific binding of non-targeted proteins to the affinity matrices or by physiologically relevant interactions between targeted and non-targeted proteins [15, 29–33]. In the case of SuperMix partitioning, the column contains an undefined mixture of antibodies in differing amounts. We anticipate that many proteins may partially bind to the column due to the presence of antibodies, as well as to interactions with other MAP and LAP. Therefore, we suggest that the use of the SuperMix column be considered as a fractionation scheme rather than a depletion scheme so that all proteins will be recovered in either the bound or flow-through fractions. In the case of partial binding to the columns, we have demonstrated that the extent of nonspecific or specific binding for a given protein is reproducible and quantifiable [6, 15].

Another valid concern is that LAP in the bound fraction will be difficult to detect due to the masking effects of the MAP, even if both bound and flow-through fractions are analyzed. Because of the high complexity of antibodies in the SuperMix column, we expect that some LAP of interest are partitioned predominantly to the bound fraction. In such scenarios, one might apply both single stage IgY14 separations and tandem IgY14-SuperMix separations as these two approaches afford complementary detection of LAP.

5. Concluding remarks

Enabling the removal of HAP and MAP from human biofluids, multi-component immunoaffinity separations on HPLC systems have become powerful tools for biomarker discovery studies. A number of immunoaffinity LC columns with either IgG or IgY antibodies are commercially available for removing 6 to 20 HAP in human plasma/serum depending upon the application needs. Furthermore, both HAP and MAP can be removed by applying IgY14 and SuperMix columns in tandem, which significantly improves detection of LAP and the overall proteome coverage compared to previously reported immunoaffinity separation strategies. Both single-stage and tandem IgY14-SuperMix separations are reproducible and automatable on an HPLC system, making them feasible strategies for large-scale discovery or candidate biomarker verification applications. Moreover, these

strategies can be readily integrated with any downstream LC-MS-based or gel-based approaches for protein identification and/or quantification. As a result, both single-stage and tandem IgY14-SuperMix strategies are anticipated to have broad applications in candidate disease biomarker discovery and verification studies with human biofluids.

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Abbreviations

HAP	high-abundance proteins
MAP	moderate-abundance proteins
LAP	low-abundance proteins
HSA	human serum albumin
IgG	Immunoglobulin G
SRM	selected reaction monitoring
SID	stable isotope dilution
MARS	multiple affinity removal system
IgY	immunoglobulin yolk
LC	liquid chromatography
MS	mass spectrometry

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- Immunoaffinity separations are key tools for enriching low-abundance proteins.
- Methods for IgY14 and SuperMix Immunoaffinity separations are described.
- IgY14 separation removes the top 14 abundant proteins from human plasma.
- Tandem IgY14-SuperMix separations remove up to 60 abundant proteins.
- Proteomics applications of IgY14 and SuperMix separations are highlighted.

Figure 1.

The HAP and MAP distributions in the human blood plasma proteome. The top 14 HAP are targeted by the IgY14 column (A) and ~50 MAP are targeted by the SuperMix column. The percentage of protein abundances are based on spectral count data from LC-MS/MS experiments. In this context, HAP are defined as 14 proteins captured by the IgY14 column, and MAP are those ~50 proteins captured by the SuperMix column as listed in Table 1 except C3. All other proteins are considered as LAP.

Figure 2.

The tandem IgY14-SuperMix immunoaffinity separation strategy. The SuperMix column is generated by using the protein mixture from IgY14-depleted human blood plasma as mixed antigens. For the tandem separations, plasma or other biofluid samples are initially separated by IgY14 column. The flow-through from IgY14 column is further separated by the SuperMix column into the flow-through and eluted fraction. The typical recoveries are indicated assuming ~150 μ L plasma is loaded.

Figure 3.

The IgY14-SuperMix separation strategy with the tandem connection mode. (A) IgY14 and SuperMix columns are connected to ports 1 and 2 of a six-port valve. Port 1 is connected to port 2 when human blood plasma sample is injected into the columns. The flow-through is collected as SuperMix flow-through (SuperMix_FT). (B) Port 1 is connected to port 6 following (A), and the elution buffer is injected into the IgY14 column. The eluted sample is collected as IgY14 bound (IgY14_B). (C) After IgY14_B collection, port 1 is again connected to port 2. Elution buffer is sent through the SuperMix column and the eluted SuperMix bound fraction (SuperMix_B) is collected. (D) An example of LC chromatograms of 3 QC experiments of the tandem IgY14-SuperMix separations during the initial, middle and late stage of the column life.

Figure 4.

The 40 low-abundance plasma proteins detected in 2D-LC-MS/MS analyses of SuperMix flow-through. The protein concentration is plotted on a log scale. All proteins were reported to have a normal concentration <100 ng/mL; only 21 detected by IgY-12. The protein concentrations of the two highlighted proteins, M-CSF and MMP8, were validated by ELISA assays for the same plasma sample.

Figure 5. Reproducibility of the tandem IgY12-SuperMix separations evaluated by LC-MS/MS. Reproducibility of three replicates of the tandem IgY12-SuperMix separations were assessed based on spectral count information of identified proteins from individual LC-MS/MS.

Table 1

List of moderate-abundance proteins efficiently captured by the SuperMix column*.

IPI#	Protein Name	Gene Name	Capture Efficiency (%)
IPI00164623.4	Complement C3	C3	>99
IPI00418163.3	Complement 4B	C4B	98
IPI00032258.4	Complement C4A	C4A	98
IPI00017601.1	Ceruloplasmin	CP	>99
IPI00022488.1	Hemopexin	HPX	99
IPI00019591.1	Complement factor B	CFB	99
IPI00218192.1	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	99
IPI00032291.1	Complement C5	C5	>99
IPI00006543.2	Complement factor H-related 5	CFHR5	>99
IPI00305461.2	ITH2	ITIH2	>99
IPI00515041.2	Complement factor H.	CFH	>99
IPI00292530.1	ITH1	ITIH1	>99
IPI00019580.1	Plasminogen	PLG	>99
IPI00215894.1	Isoform LMW of kininogen-1	KNG1	98
IPI00032179.2	Antithrombin III variant	SERPINC1	>99
IPI00032328.1	Isoform HMW of kininogen-1	KNG1	99
IPI00009920.2	Complement component C6	C6	>99
IPI00298828.3	Beta-2-glycoprotein 1	APOH	>99
IPI00022895.7	Alpha-1B-glycoprotein	A1BG	>99
IPI00022418.1	Fibronectin	FN1	>99
IPI00294395.1	Complement C8 beta chain	C8B	>99
IPI00291866.5	Plasma protease C1 inhibitor	SERPINC1	98
IPI00022420.3	Plasma retinol-binding protein	RBP4	>99
IPI00165421.4	SERPINC1 protein	SERPINC1	>99
IPI00021885.1	Fibrinogen alpha chain	FGA	>99
IPI00022371.1	Histidine-rich glycoprotein	HRG	99
IPI00298497.3	Fibrinogen beta chain	FGB	>99
IPI00298971.1	Vitronectin	VTN	99
IPI00021891.5	Fibrinogen gamma chain	FGG	>99
IPI00736985.1	Similar to ceruloplasmin	LOC441368	>99
IPI00021727.1	C4B-binding protein alpha chain	C4BPA	>99
IPI00296608.6	Complement component C7	C7	>99
IPI00011252.1	Complement C8 alpha chain	C8A	>99
IPI00022391.1	Serum amyloid P-component	APCS	98
IPI00011261.1	Complement C8 gamma chain	C8G	>99
IPI00294004.1	Vitamin K-dependent protein S	PROS1	>99
IPI00011264.1	Complement factor H-related protein 1	CFHR1	>99
IPI00006154.1	Complement factor H-related protein 2	CFHR2	>99
IPI00021364.1	Properdin	CFP	93

IPI#	Protein Name	Gene Name	Capture Efficiency (%)
IPI00022392.1	Complement C1Q subunit A	C1QA	>99
IPI00022394.2	Complement C1Q subunit C	C1QC	>99
IPI00477992.1	C1Q subcomponent, B chain	C1QB	>99
IPI00027507.1	Complement factor H-related protein 3	CFHR3	>99
IPI00025862.1	C4B-binding protein beta chain	C4BPB	>99
IPI00029168.1	Apolipoprotein(a)	LPA	>99

* The capture efficiency was estimated for each protein based on MS/MS spectral count data by dividing the spectral count for each protein from the bound fraction by the sum of spectral counts from the bound fraction (B) and flow-through fraction (FT), i.e. $B/(B+FT)$. If proteins were not observed in flow-through fraction, these proteins were represented as >99%.