A New Disposable, Specific Ligand-Based Method for Depletion of Albumin and IgG From Serum and Plasma

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Abstract

Over the past decade the interest in proteomics research has increased dramatically. However, meaningful proteomics data relies on the development of reproducible and rapid methods, and this has been as challenging as the biological studies. Sample preparation has received a great deal of focus, with particular emphasis on plasma and serum, two of the most readily accessible biological fluids having direct clinical relevance. The protein concentrations of individual proteins in these samples range from > 10s mg/mL to < pg/mL. As a result, the most abundant proteins often mask the proteins at relatively lower concentrations. Removal of the most abundant proteins is one of the few options for scientists interested in detecting the moderate to low abundance proteins using mass spectrometry (MS) or two dimensional gel (2D gel) based proteomic analytical methods. However, the removal of abundant proteins must be balanced with the desire to minimize sample handling to limit protein loss while maintaining reproducibility and ease of use characteristics.

Pall Life Sciences has developed a new Enchant™ Multi-Protein Affinity Separation Kit for the depletion of human serum albumin (HSA) and/or human IgG. The kit utilizes a protein-based specific capture method to achieve maximal depletion of the target proteins and minimal loss of other proteins. Depletion of HSA and IgG is typically > 98%. Depletions take approximately 20 minutes from start to finish. The Enchant Multi-Protein Affinity Separation Kit Nanosep® devices loaded with HSA and/or IgG depletion resin are disposable, eliminating the possibility of cross contamination. Each column is designed for depletion of 50 µL of plasma or serum, resulting in more depleted sample per column than most other commercially available kits. The increased amount of protein allows for greater flexibility in the number and type of analyses performed after the depletion step. Since the resins are mixed by the user, it is easy to alter the ratios of HSA and IgG depletion media when working with unusual samples containing higher or lower than normal concentrations of these proteins (e.g., specific disease states). Additionally, the use of specific ligands for the capture of HSA and IgG overcomes the limitations of the commonly used blue dyes (e.g., Cibacron® blue) for albumin removal, which are known to capture many other proteins, and protein A for antibody capture, which shows widely varying degrees of affinity for the antibody isotypes and subtypes, resulting in incomplete removal of some subtypes of IgG.

Given the results presented here, we believe that the advantages of a ligand-based depletion method that is highly specific, disposable, cost effective, and flexible will make the Enchant Multi-Protein Affinity Separation Kit a strong addition to the product choices for scientists engaged in biomarker discovery and proteomics research.

Materials and Methods

Description of Serum and Plasma:
- Pooled serum and plasma purchased from Innovative Research is used for the double depletion experiments.
- Single donor plasma purchased from Seracare is used for the single depletion experiments.
- Samples were aliquoted and stored frozen until use. They were inspected for precipitates and, if necessary, centrifuged at 14,000 rpm to remove particulates.

Enchant Multi-Protein Affinity Separation Kit:
- Individually packaged resins for human albumin and IgG depletion.
- Nanosep centrifugal devices (includes reservoir for flow through).
- Bind/wash buffer for resin preparation, sample dilution, and post binding washes for those interested in the eluates.

Single Depletion of HSA or IgG:
- The serum or plasma samples are diluted 5-fold in bind/wash buffer.
- The depletion columns are prepared as per manufacturer instructions.
- The sample is added to the column and incubated, with rotation, for 15 minutes.
- The flow through fraction is collected by centrifugation 800-900 x g for 1.5-2 minutes.

Double Depletion of HSA and IgG:
- Same method as above, except that both depletion resins are added to a single Nanosep device.

Elution of HSA or IgG:
- After collection of the flow through fraction, the resin is washed 3 times with 400 µL bind/wash buffer.
- Protein is eluted from the depletion resin by incubation with 400 µL of 0.1 M Glycine pH 2.3 for 15 minutes (with rotation).
- The eluate fraction is collected by centrifugation, as above.

Enzyme-Linked ImmunoSorbent Assays (ELISA) to Measure HSA and IgG Concentration in Flow Through:
- Human Albumin ELISA Quantitation Kit and the Human IgG ELISA Quantitation Kit (Bethyl Laboratories) are used for these determinations.
- Flow through and starting samples are diluted appropriately for measurement with this kit. 100 µL of diluted sample or standard protein is used in the ELISA.
- Assays are performed according to manufacturer's recommendation except that some buffers are changed.
- ELISA plate is coated with 200 ng of the Capture Antibody per well.
- Secondary/Detection Ab is used at a 1/15,000 dilution.
- The percent of HSA and IgG depletion is calculated using the following formula:

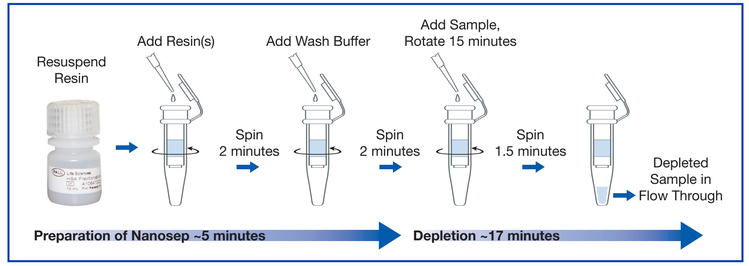
$$\frac{\text{(HSA or IgG in Start Sample)} - \text{(HSA or IgG in the FT Sample)}}{\text{(HSA or IgG in the Start Sample)}} \times 100$$

1-Dimensional SDS-PAGE:
- 2-13 µg of total protein are loaded in each lane.
- 4-12% Criterion™ XT Bis-Tris gels (Bio-Rad) run with MOPS buffer and stained with Imperial™ Protein Stain (Pierce Biotechnology).

2-Dimensional Gels of Human Plasma Before and After Depletion:
- 100-200 µg of total protein are analyzed on each gel.
- First dimension isoelectric focusing is done on a pH 3-10 non-linear ReadyStrip™ IPG strip (Bio-Rad).
- Second dimension SDS-PAGE is done with 4-12% Criterion XT Bis-Tris gels (Bio-Rad) run with MOPS buffer and stained with Imperial Protein Stain (Pierce Biotechnology).

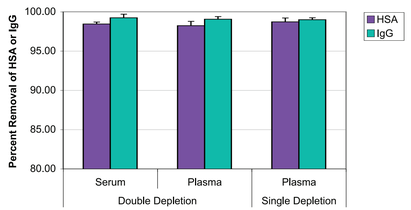
Results

Figure 1
Schematic of Depletion Method



Methods Flow Chart (Figure 1):
- A flow chart for albumin or IgG depletion for the Enchant Multi-Protein Affinity Separation Kit to highlight overall speed and ease of use.
- The double depletion has an additional spin step, adding ~2-3 minutes to the protocol time. If elution of the captured protein is of interest, 3 wash steps followed by an elution step adds ~20-30 minutes.

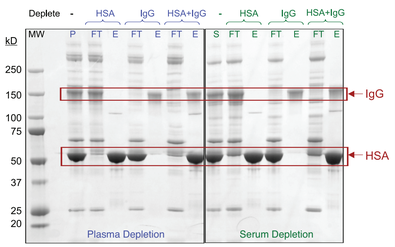
Figure 2
Percent Depletion of HSA and IgG From Plasma



HSA and human IgG are independently measured by ELISA. The average of 4-9 replicates, spanning multiple resin lots, is plotted. Error bars represent 1 standard deviation from the mean. Single donor plasma is used for single depletions. Pooled plasma or serum is used for double depletion experiments.

Percent Depletion of HSA and IgG From Singly and Doubly Depleted Plasma (Figure 2):
- ELISA data indicates very efficient depletion (> 98%) of the HSA and IgG after single or double depletion.
- The degree of HSA and IgG depletion is similar in serum and plasma.
- The single depletion results include data from 3 different lots of resin, and demonstrate very good reproducibility (standard deviation = 0.47 and 0.24 for HSA and IgG respectively). ELISA data from single and double depletions have coefficient of variation (CV) values of < 0.6% in all cases.

Figure 3
1D SDS-PAGE Analysis of Flow Through and Eluate Samples

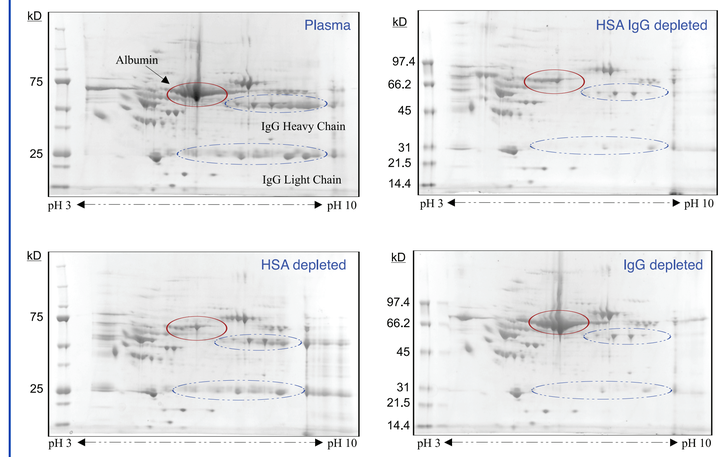


Single donor plasma and pooled serum samples are single or double depleted of HSA and IgG. The starting plasma (P) and serum (S) samples, along with depleted (FT) and eluate (E) fractions are subjected to non-reduced 1D SDS-PAGE using a 4-12% gradient gel.

1D SDS-PAGE of Flow Through and Eluate Samples (Figure 3):
- Depleted fractions (FT) from both plasma and serum are clearly depleted of the target proteins — albumin running at ~55 kDa and IgG at ~155 kDa in this non-reduced gel. Reduced gels give similar results (data not shown), except that the molecular migration (M_r) for albumin is closer to the actual MW of 66 kDa and the IgG is reduced to heavy and light chains.
- The eluate fractions (E) from single and double depletions look quite clean. There is a faint additional band above 100 kDa in the HSA eluate, but data suggests it is an albumin dimer.
- Although there is a band having approximately the same M_r as albumin in the depleted samples, reduced gel and Western blot data indicate that this is another protein.
- As expected, there are some clear differences between the plasma and serum samples, especially in the very high MW regions. These are most likely related to the loss or cleavage of proteins participating in the complement cascade activated during clot formation, which occurs during the preparation of serum.

Results (continued)

Figure 4
2-Dimensional IEF + SDS-PAGE Analysis of Plasma Before and After Single and Double Depletion



Four 2D gels of unfractionated, doubly depleted, HSA depleted, and IgG depleted plasma, respectively. 100-200 µg of total protein is used for these gels. Isoelectric focusing is done with a pH 3-10 non-linear IPG strip followed by reduced SDS-PAGE with a 4-12% Bis-Tris gel.

2D Gel Electrophoresis of Plasma Before and After Single and Double Depletion (Figure 4):
- The depletion of albumin has the greatest impact on the ability to see other proteins (single and double depletion gels).
- The depletion of IgG results in an improvement in spot visualization as well; especially in the regions where these IgG heavy and light chains normally appear (around 24 and 50 kDa). Circulating antibodies have a heterogeneous amino acid sequence which results in a range of pIs for both the heavy and light chains. Their absence clears an entire region of the gel (blue oval, +/- IgG depletion).
- The depletion of both HSA and IgG shows the greatest improvement in the ability to detect other proteins by 2D gel analysis.

Conclusions

The Enchant Multi-Protein Affinity Separation Kit is designed for the specific depletion of HSA and IgG from human plasma and serum. The challenge of abundant protein depletion for proteomic applications is the need to balance increased detection with minimal protein loss while maintaining reproducibility and ease of use characteristics. Albumin and IgG alone comprise ~80% of the total protein in serum and plasma by weight. Thus, the removal of just these two proteins can dramatically increase the detection of less abundant species while minimizing sample handling and concomitant protein loss or degradation. The commonly used blue dyes (e.g., Cibacron blue) for albumin removal are known to capture many other proteins. Protein A, used for antibody capture, shows widely varying degrees of affinity for the antibody isotypes and subtypes, resulting in incomplete removal of some subtypes of IgG. The use of specific ligands for the capture of HSA and IgG overcomes these limitations of early depletion products.

- The Enchant Multi-Protein Affinity Separation Kit efficiently and reproducibly removes > 98% of HSA and IgG from 50 µL of plasma or serum.
- The high degree of specificity of the HSA and IgG ligands for their target proteins results in very low non-specific capture of other proteins in the samples.
- The kit depletion protocol takes approximately 20 minutes to complete.
- Single-use spin columns eliminate the possibility of cross contamination.
- Minimal dilution (5 fold) and larger volume of starting sample (50 uL) allows for greater analytical flexibility and cost effective sample processing.

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