

Development of a Novel Antibody-Based Resin for the Depletion of Human Albumin and IgG

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Abstract

Antibody-based high abundance protein (e.g., albumin and IgG) depletion resins display higher specificity than dye-based resins, making them preferred over resins such as Cibacron Blue™. Antibody-based resins have much higher specificity for albumin and IgG, but typically have lower protein binding capacity. We have developed a novel high-binding capacity antibody based resin for the depletion of human albumin and IgG. The recombinant antibody ligands are small (12 kDa), single chain proteins which are expressed in yeast. The small, single chain antibody ligands produce the high specificity of conventional 150 kDa IgG antibodies but are more stable and produce a higher density of binding sites, thus increasing the antigen binding capacity of the resin.

Introduction

- The study of the human serum proteome is an area of great interest, especially the pharmaceutical potential for identifying disease biomarkers. The study of this proteome is challenging because of the wide range (10 orders of magnitude) of protein concentrations. Most of the proteins of pharmaceutical interest appear at low concentrations.¹
- A very common method for examining the proteome is two-dimensional electrophoresis (2DE) which involves the separation of the proteins first by their isoelectric point (pI) using immobilized pH gradient (IPG) strips, followed by SDS-PAGE to separate by molecular weight. The power of 2DE lies in the potential of separating several thousand protein spots on one gel. Protein spots are commonly excised, in-gel digested with trypsin and identified by MALDI mass spectrometry. Another method used for the separation of serum proteins is HPLC.^{2,3}
- Albumin and IgG's make up greater than 70% of the proteins in serum. Depletion of these high abundance proteins allows for 1) visualization of proteins co-migrating with albumin and IgG on a 1DE or 2DE gel and 2) each individual protein can be loaded at 4–5 fold higher level for improved visualization of lower copy number proteins.
- Historically, the most common method for albumin depletion is the use of Cibacron Blue resin equilibrated with a buffered salt solution. Cibacron Blue suffers from high non-specific binding and the salt interferes with 2DE. More recently, antibody-linked resins have been used to remove albumin to increase specificity but the common disadvantage is high cost and lower capacity. We have developed a novel high-binding capacity antibody based resin for the depletion of human albumin and IgG. The antibody ligands are small (12 kDa), single chain proteins which are recombinantly expressed.

Serum Facts

- Plasma = Whole Blood minus Cells
- Serum = Plasma minus Clotting Factors
- 50–70 mg/ml protein
 - Approx. 70% Albumin (35–50 mg/mL)
 - Approx. 10% IgG (5–7 mg/ml)
- At least 10,000 proteins
 - Most at very low abundance (<ng/mL)
- The concentration of Interleukin 6 (sensitive indicator of inflammation or infection, MW 21 kDa) is approx. 10 pg/ml, almost 10 orders of magnitude lower than albumin.

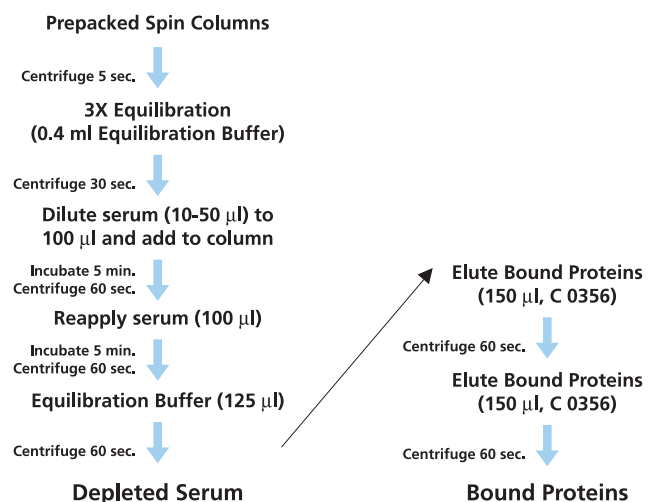
ProteoPrep® Immunoaffinity Albumin and IgG Depletion Kit (PROT-IA) for Proteomics Sample Preparation

Components for 10 serum/plasma samples (10–50 µl)

- ProteoPrep Immunoaffinity Columns, supplied as pre-packed spin columns containing 0.7 ml of a 50% novel antibody resin slurry.
- ProteoPrep Immunoaffinity Equilibration Buffer, a low ionic strength Tris-buffered solution (pH 7.4) for equilibration and washing of resin.
- Protein Extraction Reagent Type 4 (Product Code [C_0356](#)), 7.0 M urea, 2.0 M thiourea, 1% C7BzO detergent, 40 mM Trizma, for elution of bound proteins and IPG rehydration.
- 30 Collection Tubes.

Methods

Flow Chart for use of the Novel Antibody Resin



SDS-Polyacrylamide Gel Electrophoresis (1DE)

Protein samples were combined with an equal volume of 2x Laemmli Sample Buffer (Product Code [S 3401](#)) and heated at 100 °C for 5 min. The protein samples were run on 12 well 12% SDS-PAGE gels using Tris-Glycine-SDS running buffer (Product Code [T 7777](#)) and electrophoresed at 170 V. SigmaMarker™ Wide Range (Product Code [M 4038](#)) was loaded in the molecular weight marker lane. The gels were stained for 1 hr with EZBlue Reagent (Product Code [G 1041](#)) and destained with water.

Two-Dimensional Electrophoresis (2DE)

A serum sample and/or an equivalent volume of normalized depleted serum was diluted with Protein Extraction Reagent Type 4 and reduced and alkylated using PROT-RA (Tributylphosphine and Iodoacetamide). IPG strips (11 cm, pH 4–7) were rehydrated with the samples and focused overnight (60,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Product Code [I 7281](#)) and loaded onto 4–20% SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170V for 1.5 hours. The marker lanes contain SigmaMarker Wide Range. The second dimension gel was fixed and stained with EZBlue. The gels were imaged using a Fluor-S™ Multimager (BioRad). The gel images were analyzed using Phoretix 2D Expression software from Nonlinear Dynamics. Protein concentrations were determined by Bradford Assay (Product Code [B 6916](#)).

ELISA for Human Albumin and IgG

The percent depletion of human albumin and IgG was determined by ELISA. Diluted serum and depleted serum samples were added to the albumin or IgG ELISA plates coated with anti-human albumin (Product Code [A 0433](#)) or anti-human IgG (Product Code [I 2136](#)) antibodies respectively. The albumin plate was probed with mouse anti-human albumin (Product Code [A 6684](#)), followed by anti-mouse HRP conjugate (Product Code [A 9044](#)). The IgG plate was probed with anti-human IgG HRP conjugate (Product Code [A 0170](#)). The plates were developed with TMB substrate (Product Code [T 0440](#)), stopped with an equal volume of 1 M HCl and the absorption measured at 450 nm.

Albumin and IgG Depletion

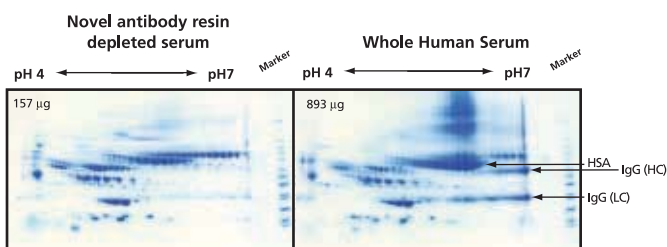


Figure 1: Albumin and IgG depletion of serum - Benefits

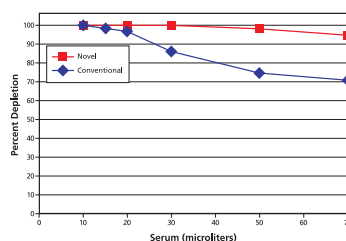
- Albumin depletion clears the upper portion of the gel, which can obscure the higher molecular weight proteins. IgG depletion clears the 50 kDa and 25 kDa regions of the gel.
- Reduces albumin and IgG contamination of spots which can interfere with MALDI identification.
- Depletion allows for increased (4-5 fold) amounts of serum that can be loaded onto a gel so that lower copy number proteins can be visualized and identified.

A 50 µl sample of human serum was depleted of albumin and IgG using the novel antibody resin (Product Code [PROT-IA](#)). Two-dimensional electrophoresis was carried out on a 15 µl serum sample and volume normalized depleted serum as described in the Methods section. The percent depletion of albumin and IgG was determined by ELISA to be 99% for both. The amount of protein loaded is detailed in the upper left hand corner of each gel.

High Depletion Capacity

Human Albumin Depletion

Panel A



Human IgG Depletion

Panel B

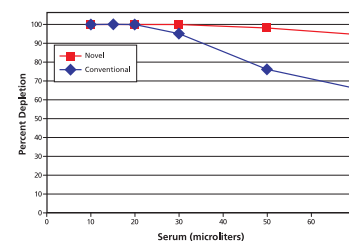


Figure 2: The novel antibody resin (PROT-IA) displays significantly higher binding capacity for human albumin and IgG than does the conventional antibody resin.

The novel antibody resin can deplete 2-4 times more serum per µl of resin than the conventional resin. For 98% depletion of albumin, the novel antibody resin will deplete 50 µl of serum where as the conventional antibody resin will only deplete 15 µl of serum. (Panel A) For 98% depletion of IgG, the novel resin will deplete 50 µl of serum where as the conventional resin will only deplete 20 µl of serum. (Panel B)

The novel antibody resin (Product Code [PROT-IA](#)) (350 µl packed resin) and a conventional antibody based resin (375 µl packed resin) were used to deplete albumin and IgG from human serum (10, 15, 20, 30, 50, 70 µl). The depleted serum from each were analyzed for residual albumin and IgG by ELISA (see Methods). The percent depletion was determined by comparison to albumin and IgG levels in "normal" (i.e. undepleted) serum.

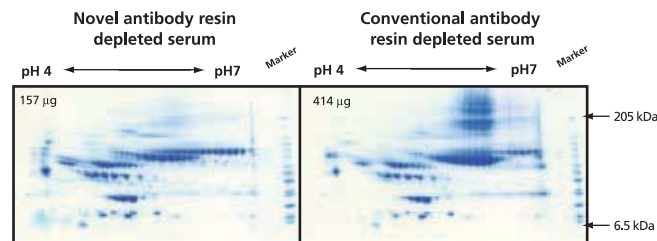


Figure 3: Increased capacity of the novel antibody resin (PROT-IA) is visually demonstrated by the large amount of albumin and IgG remaining following depletion of 50 µl serum by the conventional antibody resin.

Significantly less albumin and IgG are apparent on the gel following depletion of the sample with the novel antibody resin. This result demonstrates that the novel antibody technology has a much higher capacity for albumin and IgG than on the conventional antibody resin.

A 50 µl sample of human serum was depleted of albumin and IgG using the novel antibody resin kit (Product Code [PROT-IA](#)) following the protocol described in Methods. The final volume after depletion was 225 µl. A 50 µl sample of human serum was depleted of albumin and IgG using a conventional antibody based albumin depletion kit following the protocol included in the kit. The conventional antibody depleted serum (515 µl final volume) was acetone precipitated to remove the salts from the resin buffer and to concentrate the protein. Two-dimensional electrophoresis was carried out on volume normalized depleted serum as detailed in the Methods section.

High Specificity

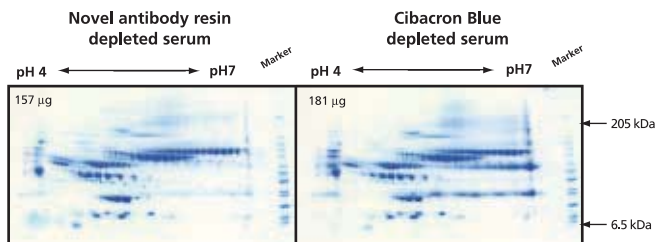


Figure 4: The novel antibody resin (PROT-IA) displays higher specificity than the Cibacron Blue based resin kit.

The 2DE analysis software (Nonlinear Dynamics) detected 362 spots on the novel resin gel whereas 245 spots were detected on the Cibacron Blue resin gel. One hundred forty one (141) spots were detected on the novel resin gel which were not matched on the Cibacron Blue resin gel. Twenty one (21) spots were detected on the Cibacron Blue gel that were not matched to the novel gel.

Albumin and IgG were depleted from 50 µl samples of human serum using the novel antibody resin kit (Product Code [PROT-IA](#)) following the protocol detailed in the Methods section. Albumin only was depleted from 50 µl of human serum using a commercially available Cibacron Blue based albumin depletion kit. Two-dimensional electrophoresis was carried out on volume normalized depleted serum as detailed in the Methods section. The protein amount loaded is detailed in the upper left hand corner of each gel.

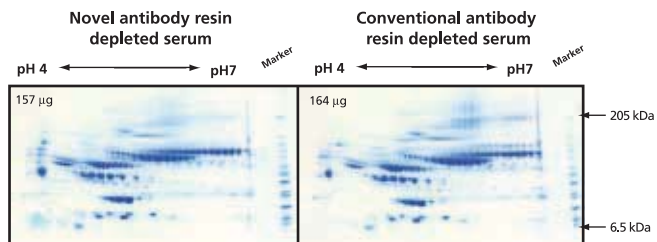


Figure 5: The novel antibody resin (PROT-IA) displays higher specificity than a conventional antibody-based resin kit.

The 2DE analysis software (Nonlinear Dynamics) detected 362 spots on the novel resin gel whereas 279 spots were detected on the conventional antibody resin gel. Ninety three (93) spots were detected on the novel resin gel which were not matched on the conventional resin gel. Only seven (7) spots were detected on the conventional gel not matched to the novel gel.

Albumin and IgG were depleted from 50 µl of human serum was using the novel antibody resin kit (Product Code [PROT-IA](#)) following the protocol detailed in the Methods section. The final volume of the depleted serum was 225 µl. Albumin and IgG were also depleted from 15 µl of human serum using a commercially available conventional antibody based albumin depletion kit following the protocol included in the kit. The depleted serum (515 µl final volume) obtained from the conventional antibody resin was acetone precipitated to remove the salts and to concentrate the proteins. Two-dimensional electrophoresis was carried out on volume normalized depleted serum (equivalent to 15 µl serum) as described in the Methods section. The protein amount loaded is detailed in the upper left hand corner of each gel.

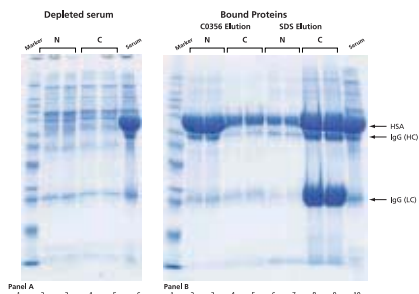


Figure 6: Removal of bound proteins from the novel resin to confirm specificity uses 2DE compatible reagents.

The specificity between the novel (N) antibody resin (Panel A, lanes 2 and 3) and the conventional (C) antibody resin (Panel A, lanes 4 and 5) appears to be comparable as seen in the depleted serum samples. The bound proteins are more easily removed from the novel antibody resin (Panel B, lanes 2, 3, 6 and 7) using IEF compatible reagents containing urea, thiourea and C7BzO detergent (Product Code [C_0356](#)). Following extraction with [C_0356](#), the resins were boiled in Laemmli sample buffer containing sodium dodecyl sulfate (SDS) (Panel B, lanes 8 and 9). The linkage of the antibodies to the novel antibody resin appears more stable as displayed by the large amount of heavy and light chain antibody subunits cleaved from the conventional resin.

Albumin and IgG were depleted from 50 µl of human serum using the novel antibody resin (Product Code [PROT-IA](#)) following the protocol detailed in Methods section (Panel A). Albumin and IgG were depleted from 15 µl of human serum using a commercially available conventional antibody based albumin depletion kit following the protocol included in the kit. Samples of the novel antibody depleted serum (lanes 2 and 3) and conventional antibody depleted serum (lanes 4 and 5) loaded onto the 12% SDS-PAGE gel were volume normalized to 0.3 µl of serum (lane 6).

Following depletion, the novel antibody resin (lanes 2 and 3) and the conventional antibody resin (lanes 4 and 5) were extracted with the 2DE compatible extraction reagent supplied with the PROT-IA kit (Product Code [C_0356](#)) (Panel B, lanes 2-5). The novel antibody resin (Panel B, lane 6 and 7) and the conventional antibody resin (Panel B, lanes 8 and 9) were then boiled with Laemmli Sample Buffer (Product Code [S_3401](#)) at 100 °C for 5 min. The samples loaded were volume normalized to 0.3 µl of serum (Panel B, lane 10).

Conclusions

- The novel small, single chain antibody resin has 2-4 times the serum capacity compared to conventional antibody-based resins.
- The novel antibody based resin has higher specificity than the Cibacron Blue resin and the conventional antibody based resin.
- Buffers have been utilized that are directly compatible with 2DE, thereby eliminating the need for protein precipitation procedures prior to electrophoresis.
- The novel antibody based depletion resin facilitates fast, efficient and specific removal of albumin and IgG from serum.

References

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2. Adkins, J.N., et al., Toward a Human Blood Serum Proteome, *Mol. Cell. Proteomics*, **1**, 947-955 (2002).
3. Rengarajan, K., et al., Removal of Albumin from Multiple Human Serum Samples, *BioTechniques*, **20**, 30-31 (1996).

