

Identification Of Human Serum Proteins By 2DE And MALDI Mass Spectrometry Otherwise Masked By Albumin Using The ProteoPrep™ Blue Albumin Depletion Kit



SIGMA-ALDRICH

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Abstract

Dye-based albumin depletion resins (e.g. Cibacron Blue) have several advantages over antibody-based resins including 1) higher binding capacity and 2) lower cost. One common disadvantage with Cibacron Blue-based resins is higher non-specific binding. The ProteoPrep Blue Albumin Depletion Kit uses a proprietary small ligand resin with optimized binding and washing conditions to significantly reduce the level of non-specific binding seen with Cibacron Blue-based resins. Many available albumin depletion products have salts (e.g. 150 mM NaCl) in the equilibration and wash buffers. Depleted serum using salt-based buffers typically requires a precipitation step in order to remove the salts prior to 2DE for well-focused gels. Precipitation is time consuming and has the potential risk of loss of protein. In this poster, it is shown that urea can be used to replace salts and will allow for albumin and IgG's to bind to the resin while inhibiting non-specific binding. Removal of albumin and IgG from serum allows for the visualization and identification of low abundance proteins with similar pI and molecular weight, which were previously masked. Removal of these high abundance proteins also allows for a greater (e.g. 5 times) proportion of serum to be applied to the IPG strips, thus increasing the chance for identification of low abundance proteins.

Introduction

- The study of the human serum proteome is an area of great interest, especially the pharmaceutical potential for identifying disease biomarkers. A challenge for studying this proteome is the wide range (10 orders of magnitude) of protein concentrations. Most of the proteins of pharmaceutical interest appear at low concentrations¹.
- One of the most popular methods 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 the serum proteins is HPLC^{2,3}.
- Albumin and IgG's make up greater than 70% (by weight) of the proteins in serum. Depletion of these proteins allows for 1) visualization of proteins co-migrating with albumin and IgG on a 1DE or 2DE gel and 2) higher sample load (4 to 5 fold) for improved visualization of lower copy number proteins.
- Historically, the most common method for depletion of albumin is the use of Cibacron Blue medium equilibrated with a buffered salt solution. Cibacron Blue suffers from high non-specific binding and the salt interferes with 2DE. Antibody-linked resins are also used to remove albumin⁴. Antibody-based resins are very specific but suffer from high cost and lower capacity. Another method involves the use of membrane-based electrophoresis systems utilizing various pH buffers and membrane size exclusion cartridges⁵. These types of systems have higher capacity but are time consuming and have lower specificity.
- Most protocols for albumin depletion of serum utilize sodium chloride or potassium chloride to reduce non-specific binding to the medium. The presence of salts can negatively impact the resolution of proteins on a 2DE gel. Samples containing salts typically require precipitation of the protein sample which adds extra steps to the process and may lead to a loss of certain proteins.
- Our goal was to identify a small ligand-based medium with higher specificity than Cibacron Blue for albumin and also remove the need for salt(s) in the buffers.

Serum Facts

- Plasma = Whole Blood - Cells
- Serum = Plasma - Clotting Factors
- 50 - 70 mg/ml protein in serum
 - Approx. 70% Albumin (35-50 mg/ml)
 - Approx. 10% IgG (5-7 mg/ml)
- At least 10,000 proteins
 - Most at very low abundance (< 1 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 less than albumin.

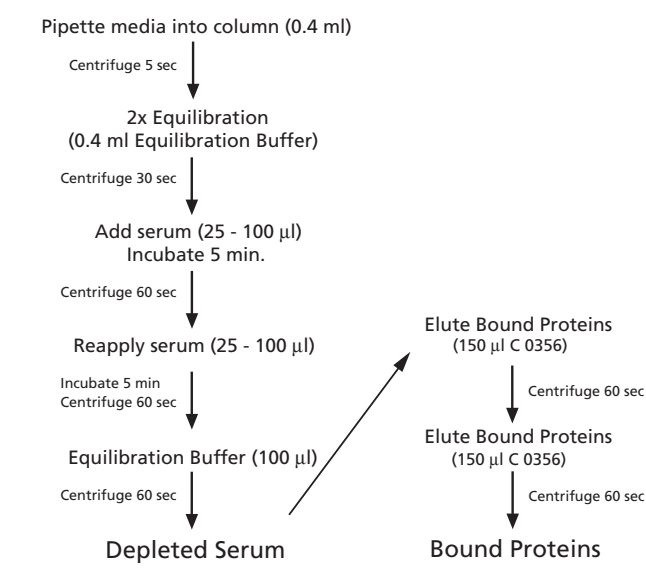
ProteoPrep Blue Albumin Depletion Kit (PROT-BA)

Components for 25 serum/plasma samples (25-100 µl)

- ProteoPrep™ Blue Albumin Depletion Medium (Product No. P 1120), supplied as a 10 ml suspension containing 60% packed mixture of two medias: a blue ligand conjugated to an agarose base medium and Protein G agarose medium.
- ProteoPrep™ Blue Equilibration Buffer (Product No. P 1245), a low ionic strength Tris-buffered urea solution (pH 7.8) for equilibration and washing of medium.
- Protein Extraction Reagent Type 4 (Product No. C 0356), 7.0 M urea, 2.0 M thiourea, 1 % C7BzO detergent, 40 mM Trizma, for elution of bound proteins and IPG rehydration.
- 25 Spin Columns and 75 Collection Tubes

Methods

Albumin Depletion with ProteoPrep Blue



SDS-PAGE (1DE)

Protein samples were combined with an equal volume of 2X Laemmli Sample Buffer (Product No. S 3401) and heated at 100°C for 5 min. The protein samples were run on 12 well 4-20% SDS-PAGE gels using Tris-Glycine-SDS running buffer (Product No. T 7777) and electrophoresed at 170 V (constant voltage). SigmaMarker Wide Range (3.5 µl of Product No. M 4038) was loaded in the molecular weight marker lane. The gels were fixed and stained for 1 hr with EZBlue stain (Product No. G 1041).

Two-Dimensional Electrophoresis (2DE)

A serum sample and/or an equivalent volume of normalized depleted serum were diluted with Protein Extraction Reagent Type 4 (Product No. C 0356) and reduced and alkylated using PROT-RA (Tributyl Phosphine and Iodoacetamide). IPG strips (Product No. I 2906, 7 cm, pH 4-7, Product No. I 2531, 7 cm, pH 3-10, or Product No. I 3531, 11 cm, pH 4-7) were rehydrated with the samples and focused overnight (7 cm - 60,000 Vhr, 11 cm - 85,000 Vhr). The strips were equilibrated for 15 min with IPG Equilibration Buffer (Product No. I 7281) and loaded onto 4-20% SDS-PAGE gels with IPG wells. The gels were electrophoresed at 170V (constant voltage). The marker lanes contain SigmaMarker Wide Range (Product No. M 4038). The second dimension gel was fixed and stained with EZBlue (Product No. G 1041).

In-Gel Digestion and MALDI Identification

Coomassie-stained spots on the gels were excised and processed using the Trypsin Profile IGD Kit (Product No. PP0100). Briefly, the gel plugs were destained and dried. The dried plugs were then rehydrated with Proteomics Grade Trypsin and digested overnight at 37°C. The extracted peptides were dried down and then redissolved with the MALDI matrix of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, with 0.03% trifluoroacetic acid. The digested samples were analyzed on a Kratos AXIMA CFR plus MALDI TOF mass spectrometer in positive ion reflection mode, with spectrum acquisition achieved via the summation of 100 shots per sample. Protein identification was performed using peptide mass fingerprint database interrogation of the NCBI database employing MASCOT.

ELISA Assays for Human Albumin and IgG

The percent depletion of human albumin and IgG was determined by ELISA assays with 90 min incubations at 37°C. Diluted serum and depleted serum samples were added to plates coated with capture antibodies. The albumin plate was probed with primary antibodies for albumin, followed by an HRP conjugate antibody for the primary. The IgG plate was probed with a primary antibody conjugated with HRP. The plates were developed with TMB substrate (Product No. T 0440). The reaction was stopped with an equal volume of 1 M HCl and measured at 450 nm wavelength.

Effect of Salt on 2DE of Serum Proteins (Albumin and IgG Depleted)

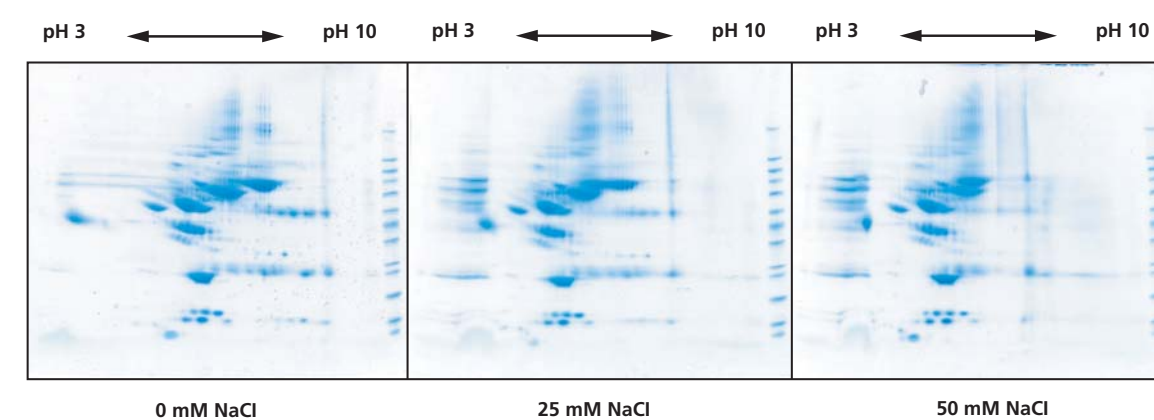


Figure 1. This figure demonstrates the negative impact that the presence of salts have on the resolution of proteins on a 2DE gel.

- Increasing salt concentration causes lack of protein focusing on the high pH end of the first dimension.
- The proteins that do focus tend to shift toward the low pH end and are less resolved.
- A subset of the proteins stack up on the low pH end of the strip.

A 75 µl sample of human serum was depleted of albumin and IgG using the ProteoPrep Blue Albumin Depletion Kit. Two-dimensional electrophoresis was carried out on depleted serum using 7 cm, pH 3-10 IPG strips as described in the Methods section, with no salt - or in the presence of either 25 or 50 mM NaCl.

Benefits of Albumin/IgG Depletion of Serum for 2DE

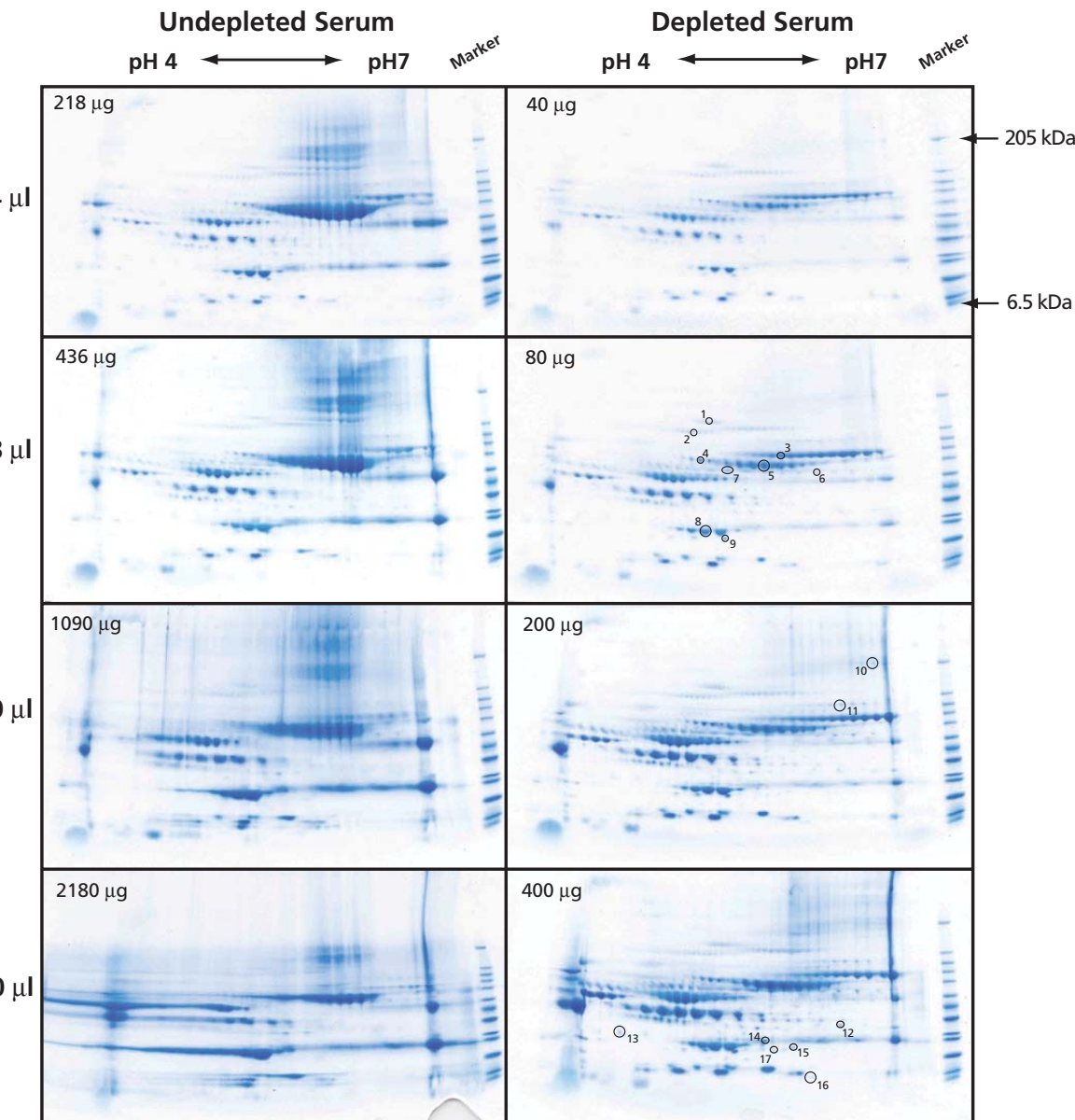


Figure 2. Albumin/IgG depletion of serum for 2DE shown at various load volumes. The 2DE gels show the benefits for albumin and IgG depletion of serum, namely:

- Exposure of proteins otherwise masked by albumin and IgG
 - The kit allows for significantly higher loading of depleted proteins and thus detection of low copy proteins.
- Depletion cleared the upper right portion of the gel, which can obscure the higher molecular weight proteins (e.g. spots 1-4).
 - Depletion allowed for increased (4-5 fold) amounts of serum that can be loaded onto a gel so that lower copy number proteins could be visualized and identified (e.g. spots 13-17).
 - Approximately 400-500 µg of total protein can be loaded onto an 11 cm strip before significant smearing and reduction of absorbed proteins is evident.

A 50 µl sample of human serum was depleted of albumin and IgG using the ProteoPrep Blue Albumin Depletion Kit. Two dimensional electrophoresis was carried out on 4, 8, 20 and 40 µl undepleted serum samples and volume normalized depleted serum using 11 cm, pH 4-7 IPG strips as described in the Methods section. The percent depletion of albumin and IgG was determined by ELISA to be 98% and 94% respectively. The numbered arrows indicate spots which were in-gel digested and putatively identified using MALDI, (see Table 1).

Table of Proteins Putatively Identified Using MALDI Mass Spectrometry.

Spot Number	Protein Identification	% Coverage
1	Ceruloplasmin	21
2	Inter-alpha-trypsin inhibitor	30
3	Transferrin	63
4	Alpha-1-β-glycoprotein	32
5	Albumin	62
6	Alpha-2-macroglobulin	30
7	FcγRI (CD89)	53
8	Apolipoprotein A-1	70
9	Retinol Binding Protein	50
10	Transferrin	27
11	Complement Factor B	46
12	Ficolin 3	47
13	Apolipoprotein D	38
14	Serum Amyloid P Component	53
15	Plasma Glutathione Peroxidase	54
16	Haptoglobin alpha chain	29
17	BCL2-related protein A1	62

Table 1. Putative Protein Identification

The table of proteins above were putatively identified using MALDI mass spectrometry.

- Several proteins were identified which would have otherwise been difficult to detect at lower sample loads.
- Spot #17; BCL-2 related protein A1 - Apoptosis Inhibitor
- Spot #14; Serum Amyloid P Component - Constituent of the abnormal tissue deposits in amyloidosis, including Alzheimers disease.
- Spot #13; Apolipoprotein D - Involved in neuroregenerative and neurodegenerative processes, and is upregulated in late-onset Alzheimers disease.
- Spot #12; Ficolin 3 - Indicator for Lupus Erythematosus

Urea-based vs Salt-based Buffers

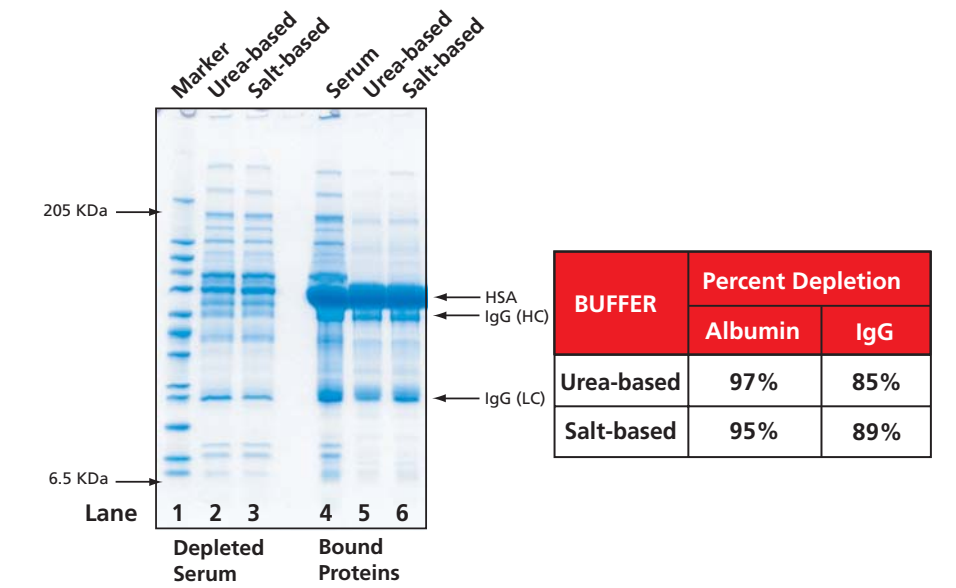


Figure 3. Replacing salt with urea maintains albumin and IgG binding

Salt (e.g. 150 mM NaCl) is effective at reducing non-specific binding of serum proteins to the resin (Lanes 3 and 6). Replacing sodium chloride with urea maintained the albumin and IgG binding of the resin (Lane 5). The urea-based buffer (Lane 5) showed slightly improved non-specific binding as can be seen by less bound proteins in the upper part of the gel compared with use of the salt-based buffer (Lane 6).

Two 75 µl samples of human serum were processed using the ProteoPrep Blue Albumin Depletion Kit equilibrated either with the supplied urea-based buffer (Lanes 2 and 5) or with 25 mM Tris-HCl, 150 mM NaCl pH 7.4 (Lanes 3 and 6). A 0.3 µl serum sample (Lane 4) and the equivalent volume normalized depleted serum and bound samples were run on 4-20% SDS-PAGE gels as described in the "Methods" section.

Evaluation of Commercially Available Kits

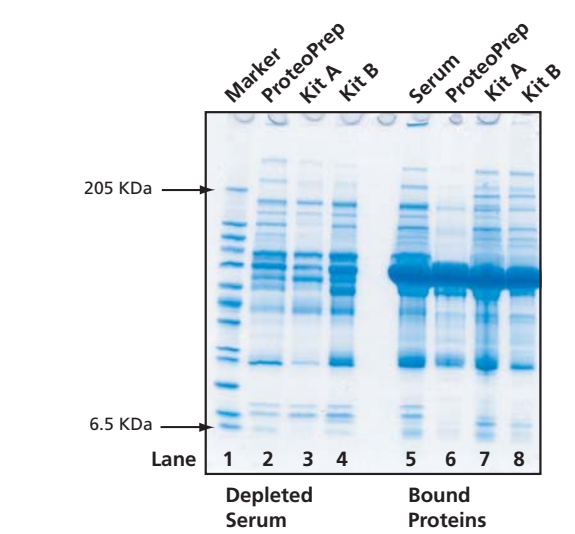


Figure 4. Existing kits typically display higher non-specific binding compared with ProteoPrep Blue

As seen in the gel, commercially available kits which use Cibacron Blue resins (Kits A and B) typically display relatively high non-specific binding for proteins other than albumin as seen in the large number of proteins bound to the resins (Lanes 7 and 8 compared to Lane 6). Kit A also contains Protein A resin for binding IgG. ProteoPrep Blue, with urea based buffers and a proprietary medium provides excellent albumin and IgG depletion (Lane 2) with low non-specific binding (Lane 6).

Each of the commercially available products (Kit A and Kit B) were used according to the protocols supplied with each kit. For the ProteoPrep Blue kit and kits A and B, 75 µl, 50 µl and 75 µl of human serum was applied respectively. The final volumes of depleted serum for the ProteoPrep Blue kit and kits A and B, were 175 µl, 400 µl and 175 µl respectively. The bound proteins were eluted from the columns using Laemmli Sample Buffer. A 0.375 µl serum sample, an equivalent volume normalized depleted serum, and bound samples were run on 4-20% SDS-PAGE gels as described in the Methods section.

Conclusions

- ProteoPrep Blue uses a unique dye-based resin which displays higher specificity for human albumin from serum than other commonly used commercially available kits and typically removes > 95% of the albumin and > 85% of the IgG.
- The ProteoPrep Blue protocol is fast, reproducible and minimizes dilution of the depleted serum.
- Depletion of albumin and IgG using ProteoPrep Blue allows for increased load of serum proteins on 2DE and subsequent identification using in-gel trypsin digestion and MALDI mass spectrometry.
- A Tris-buffered urea solution is an improvement over buffered salt solutions for albumin depletion on a dye-based resin.
- The use of the Tris-buffered urea solution removes the need for precipitation.

References

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