AlbuVoid™ Enrichment & Antibody Depletion – Tackling the Challenges of Serum Proteomics Part II

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Poster first presented at 26th International Molecular Med TRI-CON, March 10-15, 2019, San Francisco, CA USA

Abstract

Proteomic workflows that support serum proteomics can be especially challenging for two reasons: 1) the presence of highly abundant proteins, Albumin alone accounts for about 50% of the total protein mass, and 2) a particularly proteolytic resistant sample type due the large concentration of antibodies present. Many proteomic enrichment strategies employ the use of immuno-affinity depletion to remove one or more high abundance proteins. Some common limitations of immuno-affinity however are high costs, regeneration requirements which may result in a diminished and inconsistent performance, as well as a required marriage of species to antibody. Because of these limitations, researchers need ways to enrich differently. We have previously reported a variety of tools that can bias towards or against select sub-proteomes of serum without the use of immuno-affinity. Now we report the serum proteome bias characteristics of **AlbuVoid™** & **NuGel™ Protein A**, alone or in serial combination, using LC-MS reporting metrics. Products and digest conditions produce different proteome qualitative and quantitative windows of observation. For biomarker discovery, we solicit the value for enrichment of categorical sub-proteomes to provide mechanistic insight into disease pathologies. For this, a knowledgebase of over 1000 serum proteins is now available to help proteomic researchers choose the best available products and methods for their particular needs.

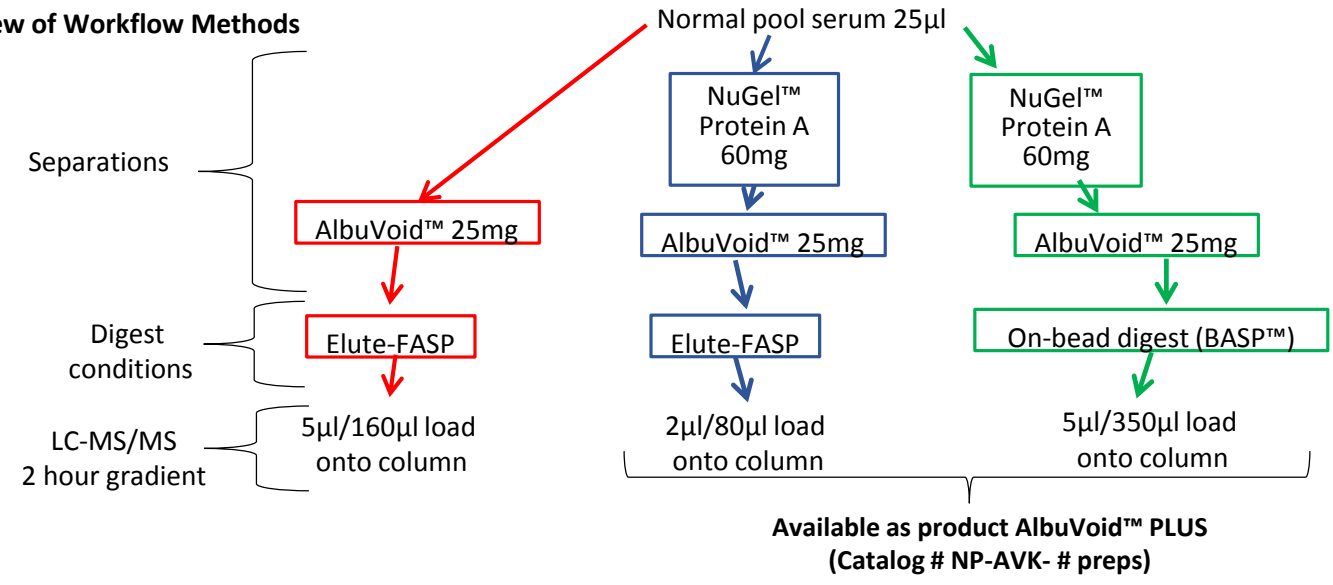
Introduction

In a previous paper, we reported on the adaptation of the BSG product **AlbuVoid™**, with a simple on-bead digestion workflow of the Albumin-depleted sub-proteome[1]. With some modest workflow adjustments, Viaralet et al. recently concluded that this method proved to be faster and more cost-effective than antibody-based methods to improve quantitative clinical proteomics[2]. We now consider the advantages of first reducing the influence of IgGs, by using a Protein A depletion, based on a NuGel™ dry powder format for which buffers adapt seamlessly to **AlbuVoid™**. With this new workflow, we compared the performance of on-bead digestion (trademarked as BASP™ for Bead-assisted Sample Prep), and off-bead (eluent workflow) using a common strong denaturing digest method conventionally called FASP.

Methods

The vast majority of the plasma proteome falls into functional categories; by mass content these are: Albumin 50-60%; Immunoglobulins 10-20%; Transport (Transferrin, Apo) 5-10%; Complement related Proteins 3-5%; Protease Inhibitors 2-5%; and all others 2-5%. While these categorical sub-proteomes are required for normal body homeostasis, they nevertheless become dysfunctional during acute-phase and chronic stimuli. Herein, we report the serum proteome bias characteristics, both qualitative and quantitative, of the products **AlbuVoid™** & **NuGel™ Protein A**, alone or in serial combination, using LC-MS reporting metrics.

Overview of Workflow Methods



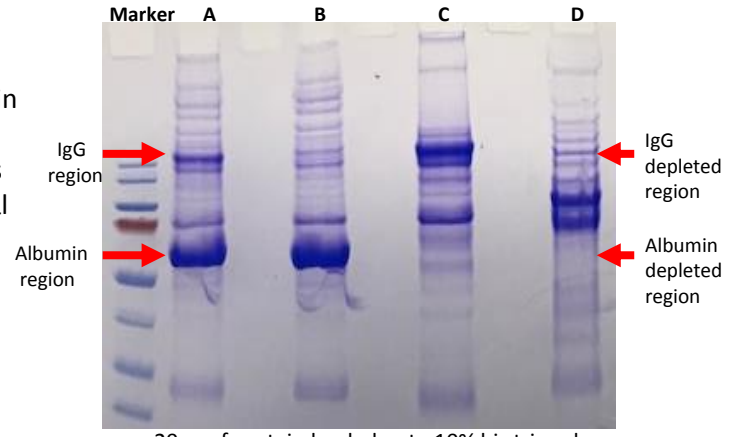
Methods Detailed

All experiments begin with 25ul of pre-filtered normal pooled serum or breast cancer serum.

For **NuGel™ Protein A**: 60mg of **NuGel™ Protein A** beads were washed with 400µl of **AlbuVoid™** Wash Buffer (AVWB supplied in kit). 25µl of serum diluted 10X with AVBB was added to washed beads. Samples were rotated for 1 hour before supernatant was collected as flow-through serum. Beads were washed with 100µl of **AlbuVoid™** Binding Buffer (AVBB supplied in kit) and the supernatant was combined with the flow-through serum, and processed with **AlbuVoid™**.

For **AlbuVoid™**: 25mg of **AlbuVoid™** beads were washed with 125µl of AVBB two times. Flow-through serum from **NuGel™ Protein A** beads or 25µl of serum, which was diluted to 300µl AVBB, was added to the washed **AlbuVoid™** beads. Samples were vortexed for 10min and the filtrate after centrifugation was discarded. Beads were then washed with 250µl of AVWB three times and the remaining low abundance proteins were either digested on-bead, or were eluted using 200µl of **AlbuVoid™** Elution Buffer (AVEB supplied in kit) for FASP digestion.

SDS-PAGE Human Serum Comparison of 3 Methods



20µg of protein loaded onto 10% bis tris gel

A – Serum neat – shows IgG and Albumin regions
B – NuGel™ Protien treated serum – shows IgG depletion
C – AlbuVoid™ treated serum – shows Albumin depletion
D – AlbuVoid™ PLUS treated serum - shows IgG and Albumin depleted regions

For FASP: To 35µl of eluent, we added 3.5µl 10X stock buffer (1M Tris pH 7.6, 1% Lauryl Dodecyl Sulphate) and 3.5µl from concentrated 1M DTT for final 100mM DTT reduction reaction at 60°C for 5 min. Samples were then diluted 1:2 with urea buffer (8M urea, 20mM methylamine hydrochloride, 100mM Tris-HCL pH 8.3) and passed through 30kDa filters (Amicon Ultra 0.5ml centrifugal filters, regenerated cellulose). Samples were washed with additional 200µl of urea buffer and then alkylated with 22.5mM of iodoacetamide in 100µl of urea buffer in the dark for 40 minutes. Samples were washed with 200µl of urea buffer three times followed by another three washes of 200µl of 50mM ammonium bicarbonate. Proteins were digested with 2µg of trypsin in 75µl 50mM ammonium bicarbonate overnight in 37°C. Flow-through was collected and the filter was washed with 200µl of 50mM ammonium bicarbonate and combined with collected peptides. 1/10 was analyzed by LC-MS.

For on-bead digest (BASP™): After bead was washed three times with 200µl of AVWB in the **AlbuVoid™** protocol, samples were reduced using 10mM DTT in 100ul AVWB. Samples were vortexed for 10 min and incubated for 30 min at 60C. After cooling to room temperature, iodoacetamide was added to 20mM and the samples were incubated for 45 min for alkylation. New collection tube was added for digestion with 8µg of trypsin in 200µl of AVWB and digested overnight at 37°C. Flow-through was collected and peptides were further extracted using 150µl of 10% formic acid. 5µl of eluate was loaded to LC-MS instrument.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

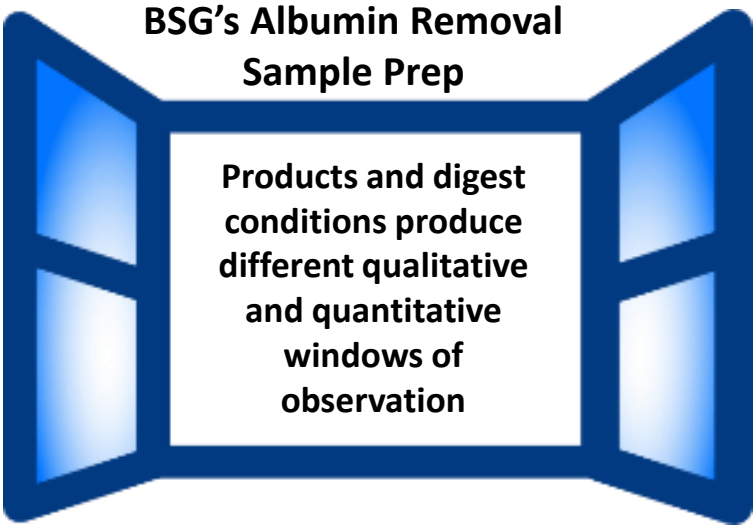
Samples were analyzed using a QExactive HF tandem mass spectrometer coupled to a Dionex Ultimate 3000 RLSCnano System (Thermo Scientific). Samples were loaded onto a fused silica trap column Acclaim PepMap 100, 75umx2cm (ThermoFisher). After washing for 5 min at 5 µl/min with 0.1% TFA, the trap column was brought in-line with an analytical column (Nanoease MZ peptide BEH C18, 130A, 1.7µm, 75µmx250mm, Waters) for LC-MS/MS. Peptides were eluted using a segmented linear gradient from 4 to 90% B (A: 0.2% formic acid, B: 0.08% formic acid, 80% ACN): 15-25%B in 40min, 25-50%B in 44min, and 50%-90% in 11 minutes. Mass spectrometry data was acquired using a data-dependent acquisition procedure with a cyclic series of a full scan with resolution of 120,000 followed by MS/MS (HCD, relative collision energy 27%) of the 20 most intense ions and a dynamic exclusion duration of 20 sec.

Database Search

LC-MSMS peak lists (.mgf files) were generated using Thermo Proteome Discoverer v. 2.1 and searched against the most up to date uniprot human database plus a database of common laboratory contaminants using an in house version of the GPM software package. Search parameters were as follows: fragment mass error, 20 ppm; parent mass error, ± 7 ppm; fixed modification, carbamidomethylation on cysteine; potential modifications during initial search, methionine oxidation and acetylation on protein N-termini; and up to one missed tryptic cleavage during the initial search and up to three missed cleavages during refinement. Potential modifications during refinement were as follows: pass 1- monoxidation at methionine and tryptophan, deamidation at asparagine and glutamine; pass 2 dioxidation at methionine and tryptophan. Maximum valid expectation scores for proteins and peptides were 0.0001 and 0.01, respectively.

Results

In the Table, we compare workflows with **AlbuVoid™** (previous and new), along with another product in our Albumin Removal catalog, that selectively binds both Albumin and IgGs, called **AlbuSorb™ PLUS**[3]. IgGs, accounting for 70-80% of the total Immunoglobulin sub-proteome are very efficiently removed by both **AlbuSorb™ PLUS** and **AlbuVoid™ PLUS**. Note that the on-bead digest methods greatly diminish the spectral counts and protein IDs associated with the Immunoglobulin sub-proteome; a particularly hard-to-digest class of proteins. In the new methods, we highlight that the Complement-related sub-proteome, being highly enriched, can be deeply investigated at the tryptic peptide level for sub-forms differentially regulated in disease.

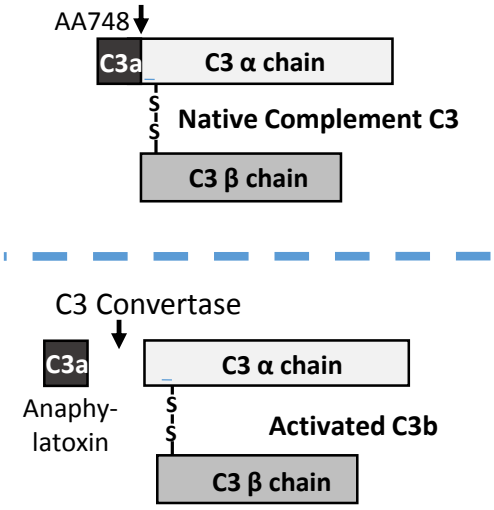


	Apprx. plasma conc.	AlbuVoid™ On-Bead 4 hr[3]	AlbuSorb™ PLUS[3]	AlbuVoid™ FASP	ProA / AlbuVoid™ / FASP	ProA / AlbuVoid™ / On-Bead
Total Spectral Counts (SC)		**8969**	**14456**	**19388**	**23575**	**23389**
Total Protein ID (≥2 SC)		**235**	**224**	**568**	**467**	**350**
% SC Albumin	**50%**	**1%**	**16%**	**3%**	**5%**	**5%**
% SC Immuno-globulins	**20%**	**15%**	**12%**	**26%**	**8%**	**6%**
% SC Apolipoproteins	**4%**	**11%**	**6%**	**4%**	**5%**	**5%**
% SC Transport Proteins	**8%**	**17%**	**22%**	**16%**	**27%**	**23%**
% SC Protease Inhibitors	**6%**	**12%**	**25%**	**6%**	**9%**	**10%**
% SC Complement related	**5%**	**28%**	**7%**	**22%**	**26%**	**31%**
% SC Coagulation/ Fibrinolysis	**4%**	**4%**	**2%**	**5%**	**4%**	**5%**
% SC Other	**3%**	**12%**	**10%**	**18%**	**16%**	**15%**

As can be seen from the Table, the Complement related sub-proteome is especially enriched with AlbuVoid™, >5X enrichment. Complement is a cascading protein interaction system that acts as an early alert and response mechanism to thwart exposure to infectious agents. Under-appreciated however, is its evolutionarily conserved link to coagulation to eliminate damaged tissues. We have previously reported this dysregulation as part of a panel of pan-cancer biomarkers called Stroma Liquid Biopsy™[4]. Observations from those studies supported many concepts surrounding the study of functional sub-forms of highly abundant proteins by differential observation at the peptide level[5].

As the highly abundant Complement related proteins exist in the circulation in a variety of functional sub-forms, we considered that our new methods might bias towards observing functional sub-forms differently. Such is the case here, where the native C3 is quantitatively different from the activated C3b sub-form when observed through the peptide features before amino acid 748 (the cleavage of C3a by C3 Convertase), and after amino acid 748.

C3 sub-forms report quantitative differences in normal vs. disease comparison with different digest methods.



	ProA / AlbuVoid™ / FASP	ProA / AlbuVoid™ / FASP	ProA / AlbuVoid™ / On-Bead	ProA / AlbuVoid™ / On-Bead
Peptide level features before AA748 (from C3 β chain)				
	Pooled Normal	**Cancer**	**Pooled Normal**	**Cancer**
# Tryptic peptide IDs (>2 SC)	**15**	**14**	**17**	**16**
Spectral Counts	**238**	**223**	**258 (1.7x cancer)**	**151**
Peptide level features after AA748 (from C3 α chain)				
# Tryptic peptide IDs (>2 SC)	**10**	**11**	**12**	**11**
Spectral Counts	**154**	**339 (2.2x normal)**	**133**	**140**

Discussion & Conclusions

Products and digest conditions produce different sub-proteome windows of observation. So, depending on the needs of the investigation, it can be valuable to consider that one or more of these categorical sub-proteomes is simply background noise whereby depletion is beneficial. While in other cases, these same categorical sub-proteomes might provide new data and information and consequently, should not be depleted. Categorically the acute-phase sub-proteomes differentiated in disease may vary greatly from those associated with chronic sub-proteomes. So there is great benefit in having options to enrich or deplete one or more of these sub-proteomes. BSG's Albumin and IgG Removal Kits[6] offer many such options:

- The '**PLUS**' products substantially deplete Immunoglobulins through separations at the protein level.
- The variable regions of Immunoglobulins are extremely heterogeneous, generating a background noise across the full LC gradient. On-bead digestion (BASP™) with **AlbuVoid™** substantially reduces the influence of such Ig peptide features. So in addition to workflow simplicity, BASP™ can be advantageous utilized in targeted proteomic workflows whenever the target proteins do not require strong denaturing conditions.
- With the exception of Immunoglobulins whereby FASP generates many more spectral features, both strong denaturing conditions (FASP) and on-bead digest (BASP™) conditions produce similar protein profiles. For certain proteins, a particular method can produce more spectral counts. So for targeted proteomics, please contact the corresponding author – Matt Kuruc, as we have a knowledgebase of over 1000 serum proteins to help select the best method(s) for particular protein(s).
- Both Apolipoproteins and heavily glycosylated proteins (i.e., α_1-Acid Glycoprotein) bind poorly to **AlbuVoid™**. For quantitative studies within these classes of proteins, **AlbuSorb™ PLUS** is recommended.
- The Complement sub-proteome is especially enriched by **AlbuVoid™ PLUS**. The digest conditions may bias towards one or more functional sub-populations, likely due to conformational transitions and protein-protein interactions (i.e., Factor Bb, Properdin) that occur upon activation. This needs further investigation.
- The low abundance sub-proteome is enriched 5+ fold with **AlbuVoid™** and 4+ fold with **AlbuSorb™ PLUS.**.

References

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