AlbuVoid™ PLUS & AlbuSorb™ PLUS Application Report
Evaluating Different Windows of Observation Solves The Many Challenges of Serum Proteomics

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Introduction

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.

With some modest workflow adjustments, Viaralet et al. recently concluded that the BSG product - **AlbuVoid™** proved to be faster and more cost-effective than antibody-based methods to improve quantitative clinical proteomics[1]. Yet high abundance depletion is only a part of the challenge, the other challenge comes from consistent trypsin digestion. In a previous paper, we reported on the adaptation of the BSG product **AlbuVoid™**, with a simple on-bead digestion workflow, herein referred to as Bead Assisted Sample Prep or simply BASP™[2].

We now consider the advantages of first reducing the influence of IgGs- a heterogeneous and proteolytically resistant class of proteins. By using a Protein A depletion, based on a **NuGel™** dry powder format, our workflow buffers adapt seamlessly to **AlbuVoid™** - supplied as a product kit called **AlbuVoid™ PLUS**. With this new method, we compared the performance of on-bead digestion), and off-bead (eluent workflow) using a common strong denaturing digest method - FASP. Likewise, we compared the performance of **AlbuVoid™ PLUS** with **AlbuSorb™ PLUS**, the product kit which depletes both Albumin & IgGs using a single step binding strategy.

There is a purpose in having two depletion strategies as each strategy can be used advantageously depending upon the goals of the investigation. For example, 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 can become dysfunctional during acute-phase and chronic stimuli.

Serum proteins circulate in the bloodstream as complexes with other proteins and often with other biomolecules, lipids, metals, cofactors, etc. So serum proteins do not circulate in isolation and will often separate and report as complexes. Therefore, having alternative enrichment strategies can provide different windows of observation to enrich and report categorical sub-proteomes. This will be especially useful to discover new biomarkers, or to refine suspected biomarkers by investigating proteoforms that arise due to, amino acid substitutions, splice variants or proteolysis. As a result, by focusing on the functional features of these protein families, proteomic researchers have opportunities to discover functional associations with disease.

Herein, we report the serum proteome bias characteristics, both qualitative and quantitative, of the products **AlbuVoid™ PLUS** and **AlbuSorb™ PLUS**, using LC-MS reporting metrics. Some examples of their selective utility for biomarker discovery in cancer are also presented. An overview of the methods used follows; the detailed LC-MS analysis having been previously reported[3-5].

Sample Prep Workflow Considerations for LC-MS Proteomics

AlbuVoid™ Plus
An Albumin & IgG Depletion reagent kit. Combines optimized immobilized Protein A with AlbuVoid™ in seamless workflow.

Key Benefit
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.

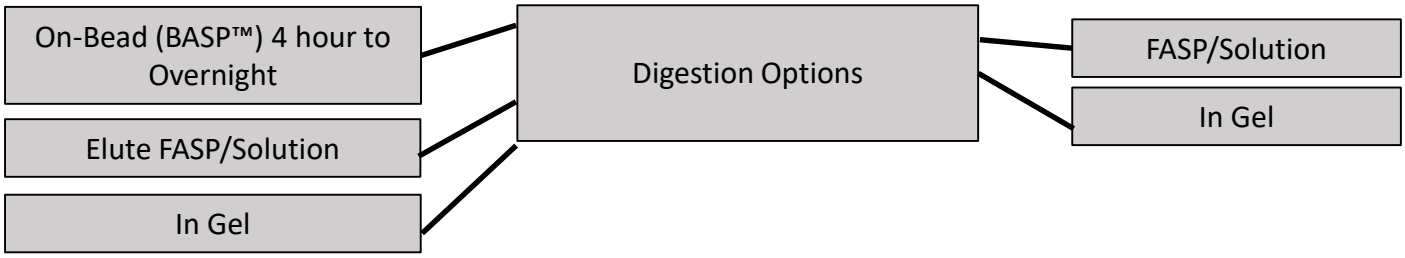
BSG's Albumin & IgG Removal Sample Prep

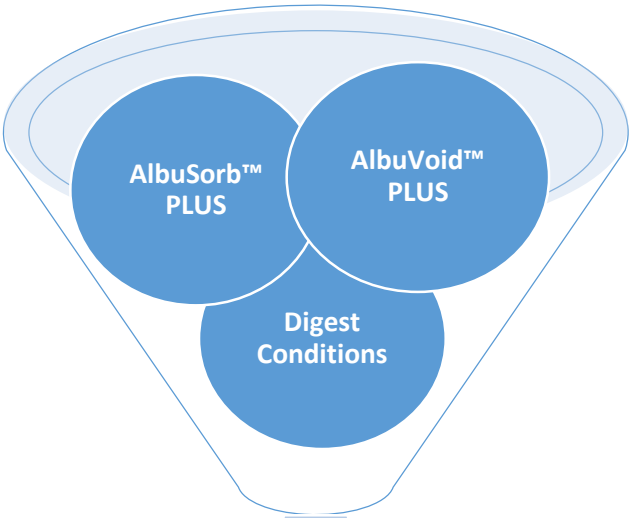
Products and digest conditions produce different qualitative and quantitative windows of observation

For more information, visit:
https://www.biotechsupportgroup.com/Albumin-Removal-s/307.htm

AlbuSorb™ Plus
An Albumin & IgG Depletion reagent kit. kit. Combines optimized immobilized Protein A with AlbuSorb™.

Key Benefit
The low abundance sub-proteome (~ng/ml) is enriched 13+ fold with **AlbuSorb™ PLUS**, uncovering unique biomarkers





Bottom Up Discovery Proteomics

Targeted Proteomics
Selection of proteotypic peptides and the optimization and validation of transitions

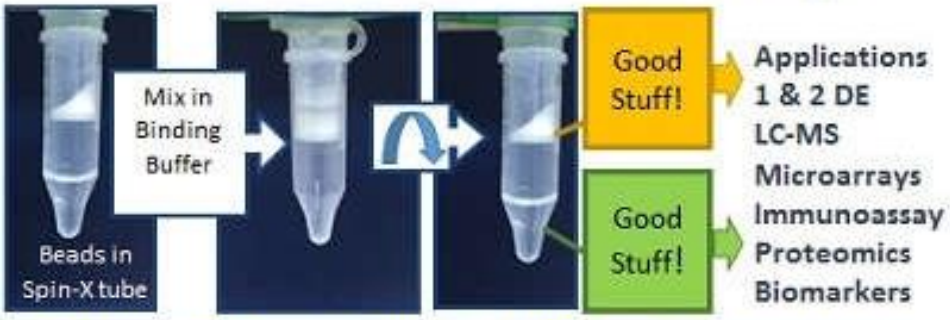
AlbuVoid™ PLUS

Optimize
- **Protein Load to Beads**
- **Digest Time**

AlbuSorb™ PLUS

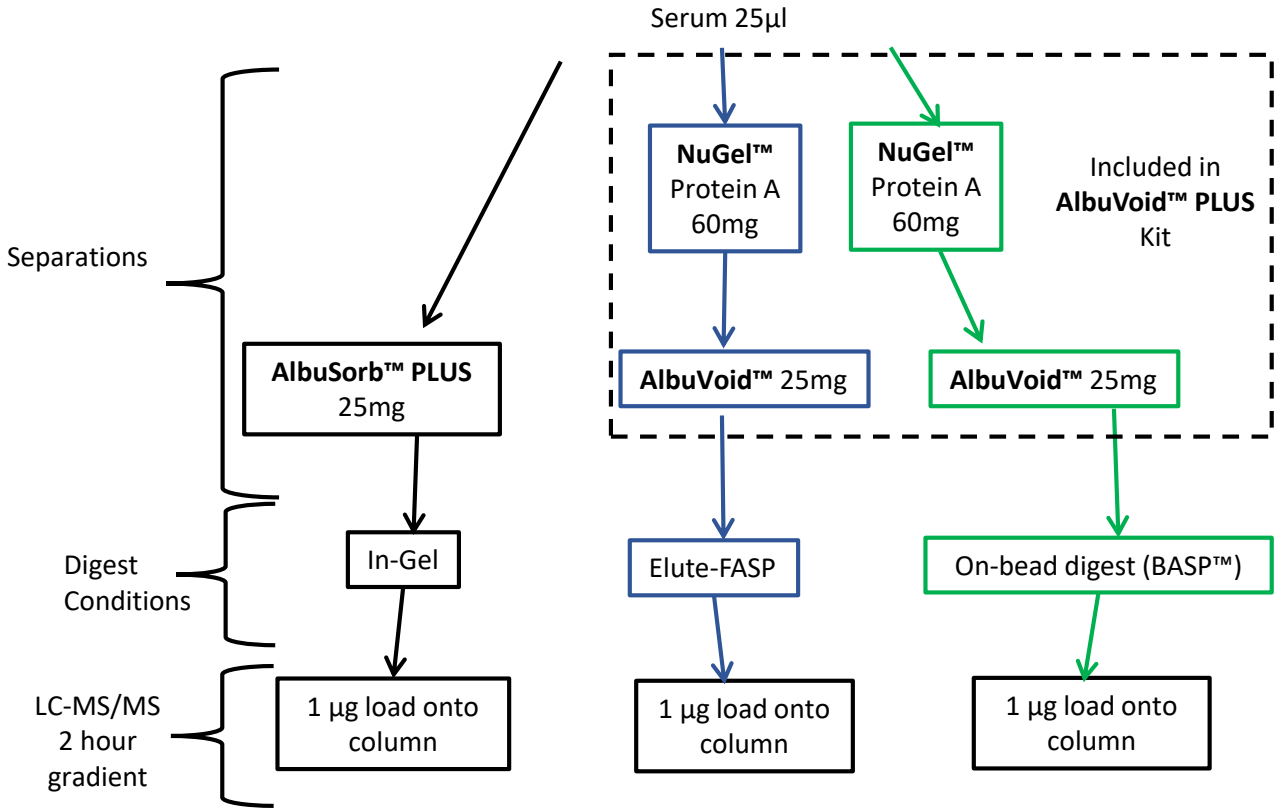
LC-MS Parameters
- **LC Gradient Profile**
- **Peptide load to LC**

Most proteomic enrichments use immuno-affinity. We don't. Here is how we enrich differently!



BSG supports two albumin and IgG depletion strategies:
Bind it or Void it

Overview of Discovery Workflow Methods



The BSG Advantage of Evaluating Different Windows of Observation

On Table 1, we compare workflows with **AlbuVoid™ PIUS** and **AlbuSorb™ PLUS**, both selectively bind both Albumin and IgGs[4]. With these two products, IgGs accounting for 70-80% of the total Immunoglobulin sub-proteome are very efficiently removed. Note that the on-bead digest methods (BASP™) diminishes 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 with **AlbuVoid™ PLUS**, can be deeply investigated at the tryptic peptide level for sub-forms differentially regulated in disease, Table 2.

Table 1	Apprx. plasma conc. %	AlbuSorb™ PLUS[3]	AlbuVoid™ PLUS / FASP	AlbuVoid™ PLUS / On-Bead
Total Spectral Counts (SC)		18890	23575	23389
Total Protein ID (≥2 SC)		370	467	350
% SC Albumin	50%	14%	5%	5%
% SC Immuno-globulins	20%	6%	8%	6%
% SC Apolipoproteins	4%	5%	5%	5%
% SC Transport Proteins	8%	18%	27%	23%
% SC Protease Inhibitors	6%	21%	9%	10%
% SC Complement related	5%	8%	26%	31%
% SC Coagulation / Fibrinolysis	4%	1%	4%	5%
% SC Other / Low Abundance	3%	26%	16%	15%

Enrichment of Protein Families

Serum proteins circulate in the bloodstream as complexes with other proteins and often with other biomolecules, lipids, metals, cofactors, etc. So regardless of the separations strategy for enrichment or depletion, serum proteins will often separate and report as complexes. Therefore, by focusing on the functional features of these protein families, proteomic researchers have opportunities to discover functional associations with disease. This has been previously demonstrated in the protease inhibitor family. Observations from those studies supported many concepts surrounding the study of functional sub-forms of highly abundant proteins by differential observation at the tryptic peptide level[6]. We highlight here 3 enrichment strategies where the product/method combination achieved several fold enrichment of the protein family. In the next section, we demonstrate how functional Complement sub-forms can be identified using this focused proteomic approach.

The BSG Advantage of Evaluating Different Windows of Observation

The Advantage of a Focused Proteomic Approach
Investigating Functional Sub-Forms of Complement C3 in Cancer

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 interconnected dysregulation as part of a panel of pan-cancer biomarkers called Stroma Liquid Biopsy™[7]. As can be seen from Table 2, the Complement related sub-proteome is especially enriched with **AlbuVoid™ PLUS**, >5X enrichment. As the highly abundant Complement related proteins exist in the circulation in a variety of functional sub-forms or proteolytically generated split products, contextually they separate as complexes.

We considered that our methods might bias towards observing functional sub-forms differently when comparing a normal population to a disease population, in this case cancer. 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. In light of these results, 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 C3 activation.

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

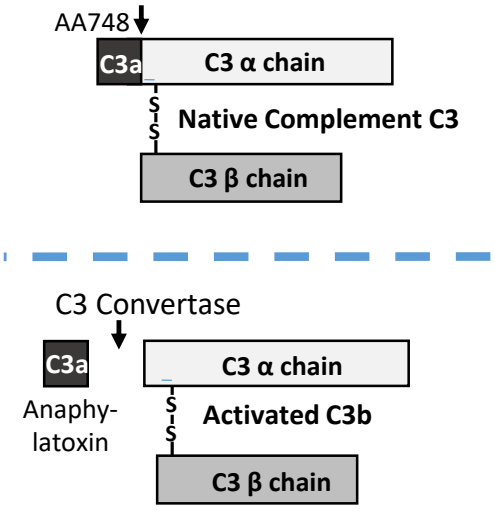


Table 2	AlbuVoid™ PLUS/ FASP		AlbuVoid™ PLUS/ BASP™	
Peptide level features before AA748 (from C3 β chain)				
	Pooled Normal	**Cancer**	**Pooled Normal**	**Cancer**
# Tryptic peptide IDs (>2 SC)	**15**	**14**	**17**	**16**
Spectral Counts	**238**	**223**	**258 (1.7x cancer)**	**151**
Peptide level features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

The BSG Advantage of Evaluating Different Windows of Observation

Enrichment of the Low Abundance Proteome

The product/method combination can also be used to study the low abundance proteome, as each produces a unique window of observation. On Table 3, we highlight representative proteins with particular spectral count bias towards either **AlbuSorb™ PLUS** or **AlbuVoid™ PLUS**. As one of these proteins (Extracellular Matrix Protein 1) is part of BSG's patent pending Stroma Liquid Biopsy™ panel[7] of cancer biomarkers, it demonstrates the advantage of evaluating multiple windows of observation.

Table 3		Spectral Counts Cancer Serum	
Protein Name	**Uniprot Identifier**	**AlbuSorb™ PLUS**	**AlbuVoid™ PLUS BASP™ On-Bead Digest**
a2-Macroglobulin subunit	sp|P01023|A2MG_HUMAN	1918	73
Haptoglobin	sp|P00738|HPT_HUMAN	1035	94
Beta-2-glycoprotein I Apolipoprotein H	tr|D9IWP9|D9IWP9_HUMAN	23	311
Pigment-epithelium-derived factor	sp|P36955|PEDF_HUMAN	4	59
Leucine-rich a2-glycoprotein	sp|P02750|A2GL_HUMAN	50	0
Extracellular Matrix Protein 1	sp|Q16610|ECM1_HUMAN[7]	0	40[7]

The BSG Advantage of Evaluating Different Windows of Observation

AlbuSorb™ PLUS identifies new potential cancer biomarkers

On Table 1 we outlined the performance of **AlbuSorb™ PLUS** compared to **AlbuVoid™ PLUS**. However as presented on Table 3, we also found particular proteins that were differentially observed by spectral counts using **AlbuSorb™ PLUS**, comparing pooled cancer serum vs. normal, shown here. These were not observed using **AlbuVoid™ PLUS** demonstrating that different windows of observation can generate different and productive discovery profiles. Of note are two classes of proteins 14-3-3 and Proteasome known to be associated with cancer.

Table 3	Gene	Uniprot ID	AlbuSorb PLUS Cancer Sp cts	AlbuSorb PLUS Normal Sp cts
Peroxiredoxin-1	PRDX1	Q06830	171	40
14-3-3 protein zeta/delta	1433Z	P63104	14	not observed
14-3-3 protein epsilon	1433E	P62258	8	not observed
14-3-3 protein gamma	1433G	P61981	3	not observed
14-3-3 protein theta	1433T	P27348	2	not observed
Chondroitin sulfate proteoglycan 4	CSPG4	Q6UVK1	7	not observed
Versican core protein	CSPG2	P13611-4	6	not observed
Proteasome subunit alpha type-7	PSA7	O14818	3	not observed
Proteasome subunit alpha type-1	PSA1	P25786	2	not observed
Proteasome subunit alpha type-2	PSA2	P25787	2	not observed
Proteasome subunit alpha type-6	PSA6	P60900	1	not observed
Proteasome subunit alpha type-3	PSA3	P25788	1	not observed
Proteasome subunit beta type-4	PSB4	P28070	2	not observed
Proteasome subunit beta type-3	PSB3	P49720	1	not observed
Proteasome subunit beta type-2	PSB2	P49721	1	not observed
Proteasome subunit beta type-6	PSB6	P28072	1	not observed
Proteasome subunit beta type-5	PSB5	P28074	1	not observed
Proteasome subunit Total			15	not observed

Discussion & Conclusions

With advantageous sample prep, LC-MS can be a very powerful and productive analytical platform for investigating the serum proteome. With efficient utilization of instrument time, we demonstrate that without immuno-affinity depletion, BSG BSG product workflows generate the capability to observe between 300-500 serum proteins. For biomarker discovery, the BSG advantage of alternative depletion and digest strategies, allows for different sub-proteome windows of observation. So, 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, like the Complement related sub-proteome, categorical sub-proteomes might provide new data and information. Consequently, these should not be depleted. 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 offer many such enrichment or depletion options:

- Without reliance on immuno-affinity depletion to remove one or more high abundance proteins, along with Albumin, the '**PLUS**' products substantially deplete Immunoglobulins through separations at the protein level.
- Though versatile to all digest methods, On-bead digestion (BASP™) in particular offers several advantages:
  - The variable regions of Immunoglobulins are extremely heterogeneous, generating a background noise across 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 to shorten digest times, and utilized in targeted proteomic workflows whenever the target proteins do not require strong denaturing conditions.
  - The seamless workflow of BASP™ is adaptable to high throughput and automation.
- 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).
- 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 (~ng/ml) is enriched 5+ fold with **AlbuVoid™ PLUS** and 13+ fold with **AlbuSorb™ PLUS**. Potential new biomarkers for cancer reportable in serum have been highlighted using **AlbuSorb™ PLUS**.
- The same consumable products and methods used for discovery can be used for targeted proteomics, making the transitional goal easier, to quantitative LC-MS clinical proteomics.

References

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