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. With some modest workflow adjustments, Viaaralet et al. recently concluded that this method proved to be faster and more cost-effective than antibody-based methods to improve quantitative clinical proteomics. 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

Separations

Digest conditions

LC-MS/MS

2 hour gradient

5µl/160µl load onto column

2µl/80µl load onto column

5µl/350µl load onto column

Available as product AlbuVoid™ PLUS (Catalog # NP-AVK- # preps)
Methods Detailed

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

For NuGel™ Protein A: 60μg 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™: 25μl 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 10 min and the filtrate after centrifugation was discarded. Beads were then washed with 250μl of AVBB 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.

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 100μl AVBW. 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™ PLUS³. 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.

<table>
<thead>
<tr>
<th>BSG’s Albumin Removal Sample Prep</th>
<th>Products and digest conditions produce different qualitative and quantitative windows of observation</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Apprx. plasma conc.</th>
<th>AlbuVoid™ On-Bead 4 hr³</th>
<th>AlbuSorb™ PLUS³</th>
<th>AlbuVoid™ FASP</th>
<th>ProA / AlbuVoid™ / FASP</th>
<th>ProA / AlbuVoid™ / On-Bead</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Spectral Counts (SC)</strong></td>
<td>8969</td>
<td>14456</td>
<td>19388</td>
<td>23575</td>
<td>23389</td>
<td></td>
</tr>
<tr>
<td><strong>Total Protein ID (≥2 SC)</strong></td>
<td>235</td>
<td>224</td>
<td>568</td>
<td>467</td>
<td>350</td>
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<tr>
<td>% SC Albumin</td>
<td>50%</td>
<td>1%</td>
<td>16%</td>
<td>3%</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>% SC Immunoglobulins</td>
<td>20%</td>
<td>15%</td>
<td>12%</td>
<td>26%</td>
<td>8%</td>
<td>6%</td>
</tr>
<tr>
<td>% SC Apolipoproteins</td>
<td>4%</td>
<td>11%</td>
<td>6%</td>
<td>4%</td>
<td>5%</td>
<td>5%</td>
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<tr>
<td>% SC Transport Proteins</td>
<td>8%</td>
<td>17%</td>
<td>22%</td>
<td>16%</td>
<td>27%</td>
<td>23%</td>
</tr>
<tr>
<td>% SC Protease Inhibitors</td>
<td>6%</td>
<td>12%</td>
<td>25%</td>
<td>6%</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td>% SC Complement related</td>
<td>5%</td>
<td>28%</td>
<td>7%</td>
<td>22%</td>
<td>26%</td>
<td>31%</td>
</tr>
<tr>
<td>% SC Coagulation/Fibrinolysis</td>
<td>4%</td>
<td>4%</td>
<td>2%</td>
<td>5%</td>
<td>4%</td>
<td>5%</td>
</tr>
<tr>
<td>% SC Other</td>
<td>3%</td>
<td>12%</td>
<td>10%</td>
<td>18%</td>
<td>16%</td>
<td>15%</td>
</tr>
</tbody>
</table>

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™. Observations from those studies supported many concepts surrounding the study of functional sub-forms of highly abundant proteins by differential observation at the peptide level⁵.
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.

### Table: Peptide level features before AA748 (from C3 β chain)

<table>
<thead>
<tr>
<th>peptide level features before AA748 (from C3 β chain)</th>
<th>Pooled Normal</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptic peptide IDs (&gt;2 SC)</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Spectral Counts</td>
<td>238</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>258 (1.7x cancer)</td>
<td>151</td>
</tr>
</tbody>
</table>

### Table: Peptide level features after AA748 (from C3 α chain)

<table>
<thead>
<tr>
<th>peptide level features after AA748 (from C3 α chain)</th>
<th>Pooled Normal</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptic peptide IDs (&gt;2 SC)</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Spectral Counts</td>
<td>154</td>
<td>339 (2.2x normal)</td>
</tr>
<tr>
<td></td>
<td>133</td>
<td>140</td>
</tr>
</tbody>
</table>

### 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 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., α₁-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 with AlbuVoid™ and 4+ fold with AlbuSorb™ PLUS.

### References