BIOTECH **SUPPORT** GROUP

Sample Prep that Matters

HEMOGLOBIN REMOVAL REFERENCE APPLICATIONS

JULY 26, 2016

Introduction

The "omics" revolution demanded new and different sample prep separations that were not efficiently performed by conventional technologies. For years the protein separations toolkit was limited to liquid chromatography and gel electrophoresis. While effective for many applications, such tools were not efficient for "omics" sample preparation, when throughput, economy and simplicity were required. Furthermore, these same separation tools most often denatured proteins which limited there use in applications which required the measurement of function or bio-activity.

HemoVoid™ derives from NuGel™ & HemogloBind™ from Polymer Chemistry

Three BSG products support Hemoglobin Removal applications:

- **HemogloBind™** & **NuGel™ HemogloBind™** for selective binding of Hemoglobin &
- **HemoVoid™** for negative selection or voidance of Hemoglobin with consequent enrichment of the remaining erythrocyte proteome on the bead

Two different surface chemistries provide the basis for our hemoglobin removal product line. First **HemoVoid™** is derived from a proprietary polymer coated, 50 µm porous silica bead surface chemistry called **NuGel™**. From this passivation platform chemistry, a library of bead architectures have been created. Each bead chemistry in the library presents a singular mixed-mode interaction; combining elements of ionic, aliphatic and aromatic hydrophobicity, and polymeric characteristics. One can think of these binding interactions in different terms; as general non-specific protein adsorbents, or as bead matrices with weak affinity or imperfect fit interactions. In this way, their binding behavior is very different from classical high affinity binding which demands near perfect fits. Under protein saturation conditions, progressive displacement provides a separation bias towards or against select proteins. As a result, all derivative **NuGel™** products were empirically characterized to meet the needs of the application; for example, **HemoVoid™** to selectively void (not bind) Hemoglobin with special binding bias towards the vast majority of the remaining low abundance erythrocyte proteome to the bead. **HemogloBind™** is different; it is a suspension product derived from a family of acid-alcohol elastomeric co-polymers. These polymers are synthesized to have separation characteristics like salts and solvents, but with the mechanical advantages of solid-phases: simple removal of the bound macromolecules with no solute carryover, and adaptability to filtration, centrifugation, and automation. **NuGel™ HemogloBind™** has similar separations performance but is supplied as a dry powder, rather than as a liquid suspension.

The BSG Advantage

All of our products have these 4 features in common:

1. *Consumable Use:* not derived from biologicals, no regeneration, cost-effective, no specialized instruments or HPLC.
2. *Functional Integrity:* retains enzymatic and biological activity for functional and chemical proteomics.
3. *Enrichment or Depletion:* strategies for both enrichment of low abundance proteomes, or depletion of high abundance proteins.
4. *On-Bead Digestion:* improves performance and workflow, unique proteolytic efficiencies.

The applications and references for use of Hemoglobin removal and depletion follows.

Bilirubin Analyses
Parvathi S. Kumar, Haree K. Pallera, Pamela S. Hair, Magdielis Gregory Rivera, Tushar A. Shah, Alice L. Werner,Frank A. Lattanzio, Kenji M. Cunnion, and Neel K. Krishna. Peptide inhibitor of complement C1 modulates acute intravascular hemolysis of mismatched red blood cells in rats.TRANSFUSION Volume 00, May 2016. doi:10.1111/trf.13674.

In brief, the study evaluated the role of a peptide inhibitor of complement C1 (PIC1) in an animal model of acute intravascular hemolysis in both prevention and rescue scenarios. The authors state "To remove free Hb that may cause optical interference in bilirubin analysis, we treated all the samples with Hb depletion from hemolyzed serum/plasma (**HemogloBind**, Biotech Support Group). Bilirubin concentration was then measured with a Bilirubin Assay Kit (Sigma-Aldrich, St. Louis, MO)."

Macromolecular Complexes
C Wan, B Borgeson, S Phanse, F Tu, K Drew, G Clark, et al. Panorama of ancient metazoan macromolecular complexes. Nature Volume:525, Pages:339–344 Date published:(17 September 2015). doi:10.1038/nature14877

Two of BSG products, **NRicher™ 6** and **HemogloBind™**, were able to contribute to this rigorous examination of protein complexes. When our products were used as a pretreatment step in the overall workflow, about twice the number of observations and annotations became possible. This further validates that the sub-proteome bias characteristics of **NRicher™ 6** can simplify complex proteomes into less complex sub-proteomes with efficiencies suitable for deep functional proteome characterization. Furthermore, this study demonstrated the importance of a key feature implicit to all of our products; that is the maintenance of functional and structural integrity after separations. Without that particular feature, these additional observations would not have been possible.

Human Peripheral Blood Mononuclear Cells (PBMCs)
Rubio-Navarro, Alfonso, et al."Hemoglobin induces monocyte recruitment and CD163-macrophage polarization in abdominal aortic aneurysm."International Journal of Cardiology (2015)."

The article states:"Conditioned mediums from AAA were incubated with **HemogloBind™** reagent for hemoglobin depletion". In brief, authors describe high infiltration of CD163 monocytes surrounding micro-vesicles, low expression of CD14+ & CD16- monocytes and high CD163 mRNA/protein expression is a feature of abdominal aortic aneurysm (AAA) molecular pathology. Healthy aorta conditioned medium or complete or hemoglobin-depleted conditioned medium from abdominal aortic aneurysm were mixed with M-CSF macrophages to track CD163 and HLA-DR expression or hemoglobin uptake.

Dried Blood Spot (DBS)/Whole Blood
Hakuna, Lovemore, et al. "A simple assay for glutathione in whole blood."*Analyst* (2015). (http://pubs.rsc.org/en/content/articlelanding/2015/an/c5an00345h)

This research article cites **HemogloBind™** to deplete hemoglobin (Hb) from whole blood samples. Using a resorufin-acrylate fluorescent probe, GSH is quantitated in deproteinized blood plasma and whole blood samples. The article states: "Apart from dilution, Hb can be removed using a commercial product, **HemogloBind™**, which can isolate and remove up to 90% of blood Hb." Glutathione (GSH) is an antioxidant involved on nitric oxide regulation, covalent hemoglobin binding, DNA binding, leukotriene synthesis, protein synthesis and sepsis pathways. GSH is elevated in cancer tissues. Dried blood spot sampling is minimally invasive GSH and can allow further research on neurodegenerative diseases, chronic respiratory diseases, cancer and diabetes.

Blood Plasma
Johns, Michael, et al. "SR-135, a peroxynitrite decomposing catalyst, enhances β-cell function and survival in B6D2F1 mice fed a high fat diet."*Archives of Biochemistry and Biophysics* (2015). (http://www.sciencedirect.com/science/article/pii/S0003986115001988)

A research article in the journal Archives of Biochemistry & Biophysics cites Biotech Support Group's **HemogloBind™** reagent to deplete hemoglobin (Hb) from lysed red blood cells. Authors cite peroxynitrite decomposing catalysts such as Mn(III) bis(hydroxyphenyl)-dipyrromethene complexes as important molecules in obesity sample preparation & development of anti-diabetic agents. The article states: "Blood plasma (50 μl) was mixed with HemogloBind (50 μl) to remove hemoglobin from lysed red blood cells."Plasma hemoglobin is depleted using Biotech Support Group's **HemogloBind**™ to extract hemoglobin from lysed red blood cells. Plasma concentration of high-density lipoprotein (HDL), total cholesterol and triacylglycerol (TAG) is obtained. Precipitation using polyethylene glycol (PEG) of beta-lipoproteins, very low-density lipoprotein (VLDL) and low-density lipoprotein(LDL) from HDL fractions is performed. "We are pleased with this research on **HemogloBind™**

as interference is minimized and concentration of cholesterol proteins is obtained." states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

Red Cell Lysates
Kyoungsook Park, Christopher D. Saudek, and Gerald W. Hart Increased Expression of β-N-Acetylglucosamindase (O-GlcNAcase) in Erythrocytes from Prediabetic and Diabetic Individuals.Diabetes.2010;59(7):1845-50.

Erythrocyte proteins are highly O-GlcNAcylated. In individual with pre-diabetes and diabetes, the level of O-GlcNAcase expressed significantly increases. From serum samples, erythrocyte proteins were extracted and hemoglobin was depleted followed by sonication and centrifugation. From the red blood cell lysates hemoglobin is efficiently depleted using **HemogloBind™**. Because **HemogloBind™** is engineered for a high degree of selectivity and does not cross react with most common serum components, subsequent analysis of O-GlcNAcylation process in erythrocyte proteins is done by Western blotting using an O-GlcNAc specific antibody. Finally, the study of O-GlcNAcase allows for developing, validating, and qualifying biological markers that are compared with the level of A1C.

Personal Correspondence.

"We used **HemogloBind™** successfully to remove much of the visual interference within a casein zymogram to monitor calpain activity in the Sickle red blood cells". Personal Correspondence. Athar Chishti, PhD, Professor, Department of Developmental, Molecular & Chemical Biology, Tufts University School of Medicine.

Stored Blood Products

Delobel J., Rubin O., Prudent M., Crettaz D., Tissot J.-D., Lion N. Biomarker Analysis of Stored Blood Products: Emphasis on Pre-Analytical Issues. International Journal of Molecular Sciences.2010;11(11):4601-4617

Authors Delobel et al cited **HemogloBind™**'s application for hemoglobin depletion from the paper by Alvarez-Llamas et al, A novel methodology for the analysis of membrane and cytosolic sub-proteomes of erythrocytes by 2-DE. Electrophoresis, 30: 4095–4108. For biomarker discovery from erythrocyte proteome samples of erythrocyte concentrates, platelet concentrates and fresh frozen plasma blood products are used in proteomic analysis. This paper reviews the importance of standardizing sample preparation steps and controlling pre-analytical factors to identify proteins from cytosolic or membrane fractions. After using **HemogloBind™**, one dimensional (sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE) and 2D electrophoresis are implemented to identify unique proteins from by MALDI-TOF MS analysis.

Red Blood Cells(RBC)/Forensic Research
Danielson, Phillip B. "Isolation of Highly Specific Protein Markers for the Identification of Biological Stains: Adapting Comparative Proteomics to Forensics." (2011). The National Criminal Justice Reference Service (NCJRS) has made this federally funded grant final report available electronically at: https://www.ncjrs.gov/App/Publications/abstract.aspx?ID=258706.

The report states: "Serum obtained from menstrual blood samples was typically contaminated with erythrocyte cellular components due to the lysing of fragile red blood cells that are abundant in the endometrial lining during menses. As the serum from menstrual blood samples contained large quantities of hemoglobin which served to mask the detection of less abundant menstrual blood specific proteins. For this reason, hemoglobin was removed from collected serum prior to proteome fractionation through use of **HemogloBind™** (Biotech Support Group, Monmouth Junction, NJ). **HemogloBind™** does not cross react with most common serum components, making it suitable for the proteomic applications of this research project".

Red Blood Cells (RBC)/ Whole Blood
Snider, Thomas H., Christina M. Wilhelm, Michael C. Babin, Gennady E. Platoff Jr, and David T. Yeung. "Assessing the therapeutic efficacy of oxime therapies against percutaneous organophosphorus pesticide and nerve agent challenges in the Hartley guinea pig." The Journal of Toxicological Sciences 40, no. 6 (2015): 759-775.

Acetylcholine is an essential neurotransmitter, and inhibitors of cholinesterases(ChEs) are potent toxins. A primary component of anti-organophosphorus therapy is an oxime reactivator to rescue inhibited acetylcholinesterases. For this, clinical signs of toxicity can be measured from blood cholinesterase [Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] activity utilizing a modified Ellman's method. Biotech Support Group's unique solid-phase polymer for hemoglobin depletion, was used for pretreatment. The article states "Briefly, whole blood samples were treated with **HemogloBind™** which interferes with the ChE activity assay due to spectral overlap."

Brittain, Matthew K., Kevin G. McGarry, Robert A. Moyer, Michael C. Babin, David A. Jett, Gennady E. Platoff, and David T. Yeung. "Efficacy of Recommended Prehospital Human Equivalent Doses of Atropine and Pralidoxime Against the Toxic Effects of Carbamate Poisoning in the Hartley Guinea Pig." International journal of toxicology (2016): 1091581816638086.

> The article states “Whole blood samples were processed and analyzed as described by McGarry et al. Briefly, whole blood samples were treated with **HemogloBind** to remove hemoglobin, which interferes with the ChE activity assay due to spectral overlap. To prepare the **HemogloBind** treated blood samples for ChE activity analysis, samples were diluted 2-fold in assay buffer (1 PBS). Subsequently, samples were diluted an additional 2-fold into the test plate by adding 100 mL of sample to a total volume of 200 mL in each well of a 96-well plate. Cholinesterase activity was assessed using a spectrophotometric assay conducted in a manner similar to Ellman et al,11 as described in the in vitro reactivation section above. The relative AChE activity level for each animal (RAAChE) was defined as the ATC turnover rate in the terminal blood sample divided by that in the same animal’s baseline blood sample. A similar calculation was performed using butyrylthiocholine (BTC) turnover rates to determine RABChE.”

Christina M. Wilhelm, Thomas H. Snider, Michael C. Babin, David A. Jett, Gennady E. Platoff Jr., David T. Yeung. A comprehensive evaluation of the efficacy of leading oxime therapies in guinea pigs exposed to organophosphorus chemical warfare agents or pesticides. Toxicology and Applied Pharmacology.
Available online 31 October 2014. doi:10.1016/j.taap.2014.10.009

> Acetylcholine is an essential neurotransmitter, and inhibitors of cholinesterases(ChEs) are potent toxins. The objective of the present study is to identify an oxime antidote, under standardized and comparable conditions, that offers protection against chemical warfare agents or pesticides. Clinical signs of toxicity were observed for 24 h post challenge and blood cholinesterase [AChE and butyrylcholinesterase (BChE)] activity was analyzed utilizing a modified Ellman's method. In the modified Ellman's enzymatic assay for evaluating ChE activity, **HemogloBind™**, Biotech Support Group's unique solid-phase polymer for hemoglobin depletion, was used for pre-treatment. The article states "Terminal blood samples were collected and processed for all survivors using **HemogloBind™**".

McGarry, Kevin G., et al. "Evaluation of HemogloBind™ treatment for preparation of samples for cholinesterase analysis." (2013). Advances in Bioscience and Biotechnology, 2013, 4, 1020-1023.

> In this article, measurement of cholinesterase activity prior to depletion and after removing hemoglobin is performed. A comparison of total cholinesterase activity with Ellman method and after **HemogloBind™** treatment prior to Ellman method did not display a statistical difference in mean ChE activity. Ellman's assay invovled measuring the sum of RBC membrane ChE activity and plasma ChE activity. Total cholinesterase activity of whole blood samples with **HemogloBind™** treatment prior to Ellman method is also consistent. Moreover, the **HemogloBind™** protocol is simpler with one incubation and short, low speed centrifugation. “This article further validates our unique surface technology approach, not based on antibodies or engineered bio-ligands, as being highly selective and an efficient method for the depletion of hemoglobin concurrent with the recovery of functional activity”, states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

Johns, Michael, et al. "SR-135, a peroxynitrite decomposing catalyst, enhances β-cell function and survival in B6D2F1 mice fed a high fat diet." Archives of Biochemistry and Biophysics (2015).

> Authors cite peroxynitrite decomposing catalysts such as Mn(III) bis(hydroxyphenyl)-dipyrromethene complexes as important molecules in obesity sample preparation & development of anti-diabetic agents. SR-135 and it's analogs are synthesized to decompose peroxynitirie. In addition, authors provide experiment data on prevention of nitration assay, rat islet uptake, quantitation of tyrosine nitration, beta-cell area quantitation, glucose-stimulated insulin secretion and plasma insulin level detection. The article states: "Blood plasma (50 µl) was mixed with **HemogloBind** (50 µl) to remove hemoglobin from lysed red blood cells."Plasma hemoglobin is depleted using Biotech Support Group's **HemogloBind™** to extract hemoglobin from lysed red blood cells. Plasma concentration of high-density lipoprotein (HDL), total cholesterol and triacylglycerol (TAG) is obtained. Precipitation using polyethylene glycol (PEG) of beta-lipoproteins, very low-density lipoprotein (VLDL) and low-density lipoprotein(LDL) from HDL fractions is performed."We are pleased with this research on **HemogloBind™** as interference is minimized and concentration of cholesterol proteins is obtained." states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

Barasa, Benjamin, and Monique Slijper. "Challenges for red blood cell biomarker discovery through proteomics." Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1844.5 (2014): 1003-1010

In brief, this review describes the many challenges to generate in-depth RBC proteome analysis, including the dynamic range problem due to hemoglobin which accounts for approximately 97% of the cytosolic mass. The article states "Hemoglobin can also be depleted from an RBC lysate by employing **HemogloBind**- [39] or **HemoVoid** [40] affinity systems. **HemogloBind™...** can as well be used to remove Hb from RBC lysates [39]. Walpurgis et al. …, **HemoVoid™**, consisting of several kinds of ionic, aromatic, and polymer ligands [40]. Low abundance proteins in the RBC lysate are captured and enriched by **HemoVoid™**, while the high abundance proteins such as Hb and CA-I are thought to quickly saturate the system, and they primarily end up in the flow-through. These Hb-depletion approaches are compatible with analysis of the RBC protein fractions by 1D or 2D gel electrophoresis, followed by protein identification through mass spectrometry." "It is worthwhile to note that the authors describe both our strategies for hemoglobin depletion, as the correct choice will vary with the application. With our own experience and with users such as those referenced in this article, we have gained the necessary knowledge to guide our users to the best option for hemoglobin depletion and/or low abundance enrichment" states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

References Acknowledged in the Review
1.[39] G. Alvarez-Llamas, F. de la Cuesta, M.G. Barderas, V.M. Darde, I. Zubiri, C. Caramelo, F. Vivanco A novel methodology for the analysis of membrane and cytosolic sub-proteomes of erythrocytes by 2-DE Electrophoresis, 30 (2009), pp. 4095–4108.

[40] K. Walpurgis, M. Kohler, A. Thomas, F. Wenzel, H. Geyer, W. Schanzer, M. Thevis Validated hemoglobin-depletion approach for red blood cell lysate proteome analysis by means of 2D PAGE and Orbitrap MS Electrophoresis, 33 (2012), pp. 2537–2545

Hikosaka, Keisuke, et al. "Deficiency of Nicotinamide Mononucleotide Adenylyltransferase 3 (Nmnat3) Causes Hemolytic Anemia by Altering the Glycolytic Flow in Mature Erythrocyte". Journal of Biological Chemistry(2014): jbc-M114.

Detection of hemolytic anemia and splenomegaly in addition to reduced adenosine triphosphate (ATP) levels was noted in cells not containing Nmnat3. Glycolysis progression was stalled at the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) step as a deficiency of Nmnat3 led to lack of co-enzyme NAD. LC-MS/MS metabolomics and stable isotope tracer analysis revealed the impact of reduced NAD quantity in RBCs on glycolysis and pentose phosphate pathway. The article states "...Each sample was normalized by hemoglobin concentration at 20 µg/µl, and then hemoglobin was depleted using **HemogloBind™".**

Alvarez-Llamas, Gloria, Fernando de la Cuesta, Maria G. Barderas, Irene Zubiri, Maria Posada-Ayala, and Fernando Vivanco. "Characterization of Membrane and Cytosolic Proteins of Erythrocytes." In Vascular Proteomics, pp. 71-80. Humana Press, 2013.

Proteomic profiling of erythrocyte proteins to identify novel proteins linked to diseases is an evolving field of clinical proteomics. Cytosolic proteins could contribute to pathology of diseased erythrocytes. Hemoglobin interferes with LC-MS/MS analysis of low abundance cytosolic proteinsAuthors cite **HemogloBind™** from Biotech Support Group for hemoglobin depletion of erythrocyte cells from the cytosolic fraction. The **HemogloBind™** protocol allows for subsequent downstream proteomic analysis using 2-DE as it reduces major interference of hemoglobin from samples of red blood cells (RBCs) and helps identify proteins.

Zihao Wang, Kyoungsook Park, Frank Comer1, Linda C. Hsieh-Wilson, Christopher D. Saudek, Gerald W. Hart. Site-Specific GlcNAcylation of Human Erythrocyte Proteins: Potential Biomarker(s) for Diabetes Mellitus. Diabetes.2008;58, 309-317.

O-GlcNAc actively cycles within erythrocytes, regulating insulin signaling and mediates glucose toxicity. Therefore studying it may reveal potential biomarker for diagnoses of diabetes. In this paper, authors studied erythrocyte proteins and compared abundances between normal and diabetic protein samples. Blood samples were obtained collected into a vial containing EDTA and OGlcNAcase inhibitor PUGNAc. Next the researchers fractionated the blood cells to isolate erythrocytes. After erythrocytes were lysed and centrifuged, the supernatant containing hemoglobin was partially depleted by **HemogloBind™**.

Datta, Pradip. Effect of Hemolysis, High Bilirubin, Lipemia, Paraproteins, and System Factors on Therapeutic Drug Monitoring. Handbook of Drug Monitoring Methods.2008; 97-109.

There are many endogenous interferents of immunoassays used in clinical laboratories which affect therapeutic drug monitoring (TDM), drugs of abuse (DAU) testing, and toxicology assays. Hemoglobin interference is caused by its absorption, fluorescence and chemiluminescence properties. In this book chapter, authors Datta et al cited **HemogloBind™**, for hemoglobin depletion and reduction of matrix effects.

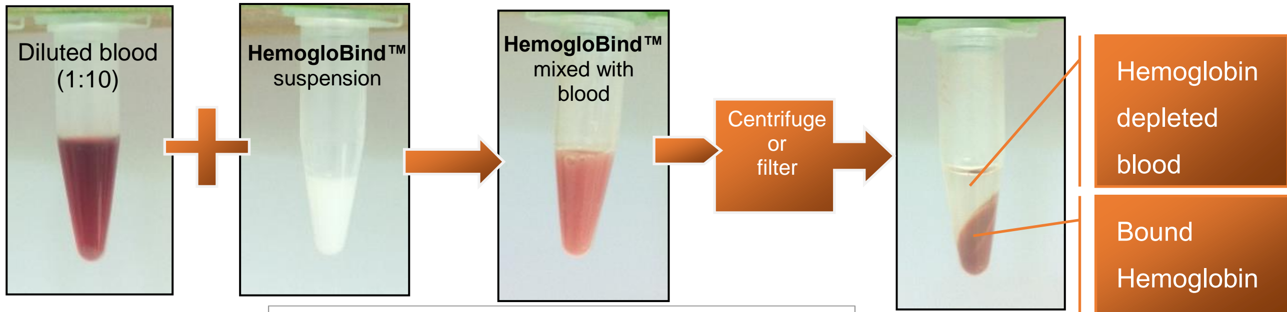
Yuichi Miki, Tomoki Tazawa, Kazuya Hirano, Hideki Matsushima, Shoko Kumamoto, Naotaka Hamasaki, Tomohiro Yamaguchi, Masatoshi Beppu. Clearance of oxidized erythrocytes by macrophages: Involvement of caspases in the generation of clearance signal at band 3 glycoprotein. Biochemical and Biophysical Research Communications.2007; 363(1):57-62

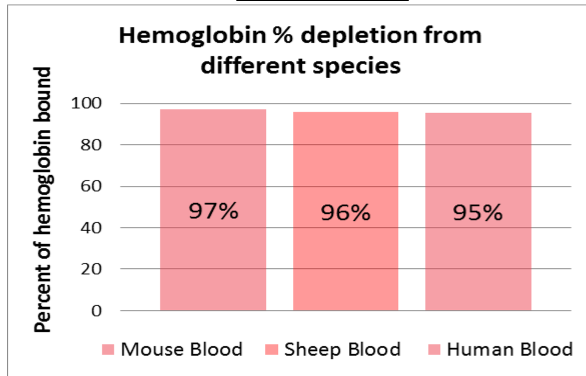
Reduction of erythrocyte clearance by macrophages happens when oxidative stress is decreased by enzymes inhibiting caspases. Band 3 aggregation increased by actions of caspases and was reduced by treatment with caspase inhibitors Z-VAD-fmk or Z-DQMD-fmk (caspase 3 selective) prior to oxidation. Pretreatment of erythrocytes exposed to H_2O_2 have increased propensity to bind and get phagocyted by macrophages. In this paper authors cite **HemogloBind**™ to remove interference.

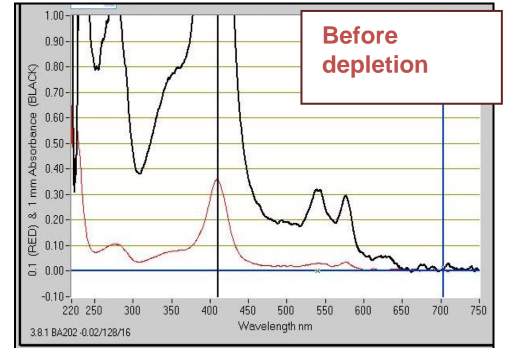
Sarawathi,et al., Relative quantification of glycated Cu-Zn superoxide dismutase in erythrocytes by electrospray ionization mass spectrometry, Biochim Biophys Acta. 1999
Feb2; 1426(3):483-90.

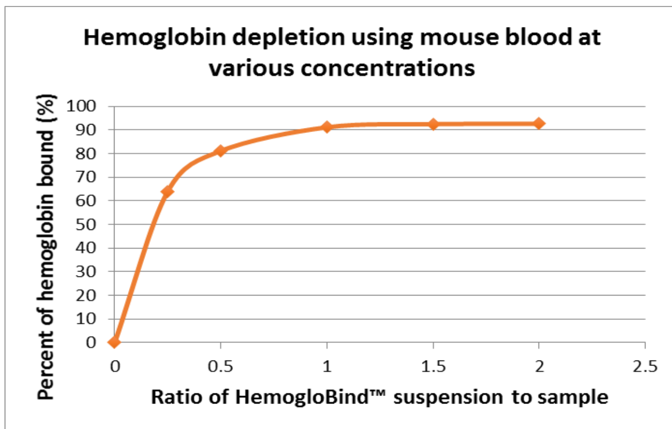
Electrospray ionization mass spectrometry (ESIMS) was used for relative quantification of glycated Cu-Zn superoxide dismutase (SOD-1) in human erythrocytes. SOD-1 samples were prepared from erythrocytes by removing hemoglobin using **HemogloBind™** followed by ethanol and chloroform extraction. The reproducibility in measurement of the relative percentage of glycated protein was good, and the standard deviation of each measurement was 4.0%. The percentage of glycation on SOD-1 was measured in 30 individuals, including patients with diabetes mellitus. The glycation levels ranged from 4.5% to below the detection limit.

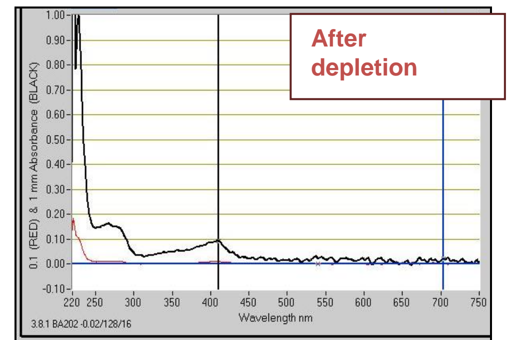
BSG Performance Characterization of **HemogloBind™** on Whole Blood Lysates.











P. falciparum clone 3D7 cultured in human erythrocytes

Lasonder E, Green JL, Camarda G, Talabani H, Holder AA, Langsley G, Alano P. The Plasmodium falciparum schizont phospho-proteome reveals extensive phosphatidylinositol and cAMP-Protein Kinase A signalling. J Proteome Research. 2012;

> Researchers discovered phosphorylated P. falciparum proteins and phosphorylation sites at the schizont stage of parasite development. Scientists found phosphorylation regulated DNA replication, transcription, translation and mitotic events (DNA packaging, chromosome organization, actin cytoskeleton). Researchers also found the CAMP-PKA signaling pathway to be involved in these events. The phosphorylation/dephosphorylation steps are important regulatory process of egress from and invasion into erythrocytes by merozoites. The analysis of Plasmodium falciparum schizont phospho-proteome involved steps which depleted hemoglobin from the samples using **HemoVoid™**. Freezing/thawing lysed P. falciparum schizont-infected erythrocytes and uninfected RBCs. Halt phosphatase and protease inhibitor cocktails were added to the sample followed by centrifugation to separate the soluble and pellet fractions. **HemoVoid™** successfully depleted hemoglobin from the soluble fraction yielding a hemoglobin depleted soluble fraction.

Lung Tissue Specimens & Pulmonary Research

Bhargava, Maneesh""Proteomic Studies in Acute Hypoxic Respiratory Failure." PhD diss., UNIVERSITY OF MINNESOTA. (2015)

> Protein expression in the bronchoalveolar lavage fluid (BALF) from subjects with acute respiratory distress syndrome (ARDS) was evaluated. The BALF samples were processed by desalting, concentration and removal of high abundance proteins. The protein fractions were trypsin digested and labeled with the iTRAQ reagent for mass spectrometry (MS). The thesis states "BALF samples containing at least 1.2 mg of proteins were processed for LCMS/MS…concentrated and desalted using Amicon 3-MWCO filters. Hemoglobin depletion was performed with **HemogloBind™** (Biotech Support Group LLC, Monmouth Junction, NJ) per the manufacturer's instructions."

Red Blood Cell Proteomics

Alvarez-Llamas, G., de la Cuesta, F., Barderas, M. G., Darde, V. M., Zubiri, I., Caramelo, C., Vivanco, F. A novel methodology for the analysis of membrane and cytosolic sub-proteomes of erythrocytes by 2-DE.Electrophoresis.2009;30:4095-4108

> Authors in this study focused on the analysis of human cytosolic and membrane sub-proteomes. Hemoglobin from samples of red blood cells was studied using different strategies for isolation of the membrane and cytosolic fractions to determine the influence it has on proteome profiling by 2-DE and hemoglobin removal. The results showed that **HemogloBind™** does have a high degree of specificity for hemoglobin and minimal interference. Particularly interesting is how authors developed a novel combined strategy based on hypotonic lysis isolation for identification of high molecular weight proteins (i.e. spectrin, ankyrin) by nano-LC coupled to an LTQ-Orbitrap mass spectrometer.

Lange, Philipp F., Pitter F. Huesgen, Karen Nguyen, and Christopher M. Overall. "Annotating N termini for the Human Proteome Project: N termini and Nα-acetylation status differentiate stable cleaved protein species from degradation remnants in the human erythrocyte proteome." *Journal of proteome research* (2014).

> This research article describes the simplicity and efficiency of BSG's proteomic sample preparation technology for depleting hemoglobin, and enriching the low abundance proteome from human erythrocyte lysates. The article states "…hemoglobin was depleted…using a commercial kit (**HemoVoid™**). This crude protein-level pre-fractionation proved helpful in identifying additional proteins and N-termini". In brief, the article describes a goal of the Chromosome-centric Human Proteome Project to identify all human protein species. With 3,844 proteins annotated as "missing" this is challenging. Enucleated and largely void of internal membranes and organelles, erythrocytes are simple yet proteomically challenging cells due to the high hemoglobin content (about 97% by mass) and wide dynamic range of protein concentrations that impedes protein identification. Using a N-terminomics procedure called TAILS, the authors identified 1369 human erythrocyte natural and neo-N-termini and 1234 proteins. From the **HemoVoid™** treated, hemoglobin-depleted soluble fraction, 778 proteins were identified, 171of which were not represented in either the soluble non-depleted fraction or the membrane fraction. This study also establishes a general workflow suitable for the in-depth determination of the position and nature of human protein N termini in different tissues and disease states. The identification of 281 novel erythrocyte proteins and six missing proteins identified for the first time in the human proteome confirmed its utility. "While the authors acknowledged other low abundance enrichment methods, our **HemoVoid™** product

was chosen for protein level enrichment. I am pleased to see it proved exceedingly useful in this exciting new area of proteomic identification." states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

Katja Walpurgis, Maxie Kohler, Andreas Thomas et al. Validated hemoglobin-depletion approach for red blood cell lysate proteome analysis by means of 2D-PAGE and Orbitrap MS.Electrophoresis.2012;

This article states the **HemoVoid™** process as a "…very efficient enrichment of low-abundant proteins by simultaneously reducing the hemoglobin concentration of the sample", and that "…a two-dimensional reference map (pH 4-7) of the cytosolic red blood cell proteome was generated and a total of 189 different proteins were identified. Thus, the presented approach proved to be highly suitable to prepare reproducible high-resolution two dimensional protein maps of the RBC cytosol and provides a helpful tool for future studies investigating disease- or storage-induced changes of the cytosolic red blood cell proteome." After using **HemoVoid™**, 2D-PAGE comparison of the unextracted hemolysate and the **HemoVoid™** depleted hemolysate displays disappearance of the prominent and smeared hemoglobin 'spot' formerly on the gel is significantly reduced. Authors recorded that **HemoVoid™** removed more than 98% of cellular hemoglobin, indirectly concentrated low abundance proteins. Upon comparison of the untreated and HemoVoid™ treated RBC lysates in the flowthrough and wash fractions by SDS-PAGE, scientists discovered the untreated RBC lysate showed two intense hemoglobin-derived bands (approximately 15 and 30 kDa), and the **HemoVoid™**-treated RBC lysate did show several bands which were not visible prior to hemoglobin depletion.

Mizukawa, B., George, A., Pushkaran, S. et al. Cooperating G6PD mutations associated with severe neonatal hyperbilirubinemia and cholestasis. Pediatric Blood Cancer.2011;56: 840-842.

The enzyme glucose-6-phosphate dehydrogenase catalyzes the first step in the pentose phosphate pathway, with concomitant reduction of the cofactor nicotinamide-adenine-dinucleotide-phosphate (NADP+) to NADPH. NADPH preserves the reduced form of glutathione and counterbalances oxidative stress in cells. G6PD deficiency is the most common erythrocyte enzyme disorder, estimated to affect more than 300 million people worldwide. The authors report a novel glucose-6-phosphate dehydrogenase (G6PD) mutation in an infant who presented with neonatal cholestasis. The article states "Blood samples from the patient and control subjects were lysed and depleted of hemoglobin using **HemoVoid™** (Biotech Support Group)". Such depletion was necessary to allow immunoblotting at an area otherwise overwhelmed by an excessive amount of hemoglobin tetramer (M.W. 68 kDa). The hemoglobin depleted fraction was analyzed by native gel electrophoresis in polyacrylamide gradient gels of 4-15%, in the absence of SDS, followed by western blotting and immunoblotting for G6PD. All three oligomers of G6PD were observed indicating that the functional and structural integrity of the enzymes were preserved after **HemoVoid™** enrichment. Bands at the expected molecular weights for the G6PD monomer, dimer, and tetramer were seen in the control specimen. The quantity of monomer was similar between patient and control, while the patient's G6PD dimer and tetramer were significantly decreased. The authors conclude that decreased amount of the enzyme along with decreased activity due to impaired oligomer formation contribute to the phenotype of class I G6PD deficiency in the patient. "This report demonstrates the features and benefits from our products that not only separate proteomes efficiently but preserve the functional and structural integrity of the proteins after separation." states Swapan Roy, Ph.D., President and Founder of Biotech Support Group.

Sudha Neelam, David G Kakhniashvili, Stephan Wilkens et al. Functional 20S proteasomes in mature human red blood cells Experimental Biology and Medicine.2011;236:580-591

HemoVoid™ is used to study the functional 20S and/or 26S proteasomes within red blood cells (RBCs; depleted of reticulocytes and leukocytes).Using methods such as double-immunofluorescence confocal microscopy to localize mature RBCs, proteasomes are isolated from mature RBC. After using **HemoVoid™**, a two-dimensional differential in-gel electrophoresis (2D-DIGE) approach was used to determine if proteasome-dependent protein degradation occurs within mature RBCs.

Walpurgis, Katja, et al. "Effects of gamma irradiation and 15 days of subsequent ex vivo storage on the cytosolic red blood cell proteome analyzed by 2D DIGE and Orbitrap MS." PROTEOMICS-Clinical Applications (2013).

Gamma irradiation of red blood cell (RBC) concentrates is routinely used to prevent transfusion-associated graft-versus-host disease. So far, the effects of ionizing radiation on RBC structure and function and especially the proteome are not fully understood. Washed RBCs were lysed, centrifuged and the **HemoVoid™** hemoglobin depletion protocol was performed. Altered protein spots and protein abundances were identified. The spotmaps of the untreated and unstored samples were compared with the samples stored following irradiation. Changes in protein abundances of the different samples was measured. Gamma irradiation was found to enhance conventional RBC storage lesions. Following 15 days of postirradiation storage, the abundances of a total of 27

spots were significantly altered and 3 out of 13 identified proteins were selected and validated as potential marker proteins for the assessment of irradiation-induced cytosolic RBC lesions.

Personal Correspondence.

***HemoVoid™** On-Bead Digestion prior to LC-MS analyses.*

The following **HemoVoid™** data was provided by Irene Granlund, Umeå University, Umeå, Sweden. It shows a comparison of Trypsin digestion of the **HemoVoid™** bound proteome, on left using on-bead digestion protocol, where the proteins are reduced and digested while they remain bound to the bead; vs. solution digestion where the proteins are first eluted from the bead and then digested using a membrane filter format.

Digestion on HemoVoid™ Matrix

Courtesy of Irene Granlund, Umeå University, Umeå, Sweden

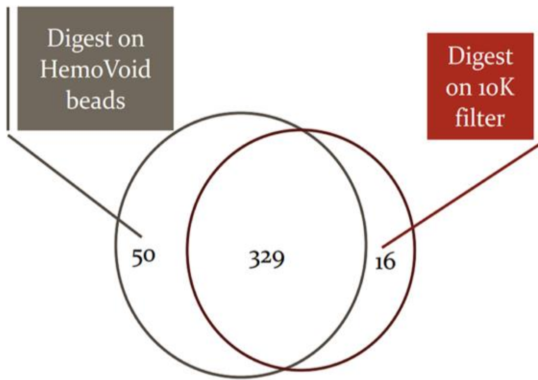
Digestion on Spin-X® tube

1. After the final wash steps from Protocol provided for HemoVoid™, add 100 µL of 5 mM DTT in HVWB to the beads for complete immersion, mix and incubate at 60°C for 1 hour.
2. Add 100 µL of 25 mM Iodoacetamide in HVWB (end concentration 12.5 mM) to the DTT/bead suspension, mix and incubate in the dark, 37°C for ½ hour.
3. Centrifuge at 3,200 x g for 2 minutes, and discard supernatant. Wash the beads with 200 µL HVWB, mix and centrifuge at 3,200 x g for 2 minutes.
4. Move the HemoVoid™ device with Hemoglobulin depleted proteins to new clean tubes.
5. Add 100 µL of digestion solution (5ng/µL trypsin in 50 mM NH_4HCO_3-solution) to the beads. Incubate 37°C, overnight (15 hours).
6. Centrifuge down at 3,200 x g for 2 minutes.
7. Wash HemoVoid™ matrix 2 times with 150 µL 50 mM NH_4HCO_3, mix and centrifuge down, at 3,200 x g for 2 minutes.

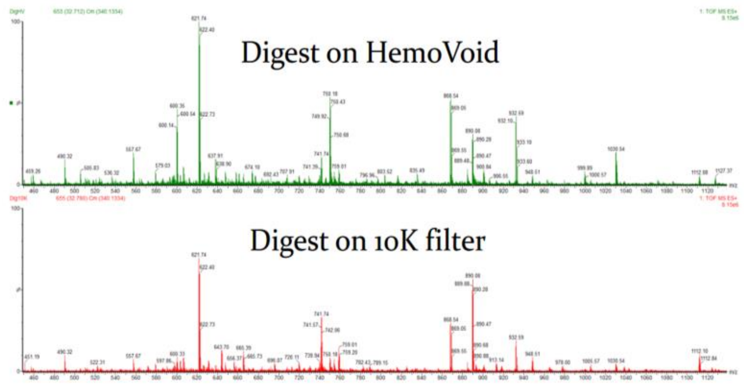
Digestion on 10K filter device

1. The proteins are eluted according to the Protocol provided with 300 µL Elution Buffer HVEB. Mixed for 10 minutes and centrifuged 4 min. at 9,000 x g.
2. The eluted sample is transferred to 10K filter device, (NanoSep 10K Omega, centrifugal device, Pall Life Sciences). The samples are centrifuged down on the filter (the solution under filter is thrown away).
3. Add 300 µL 19.5 mM DTT in Guanidine solution (6 M guanidine, 0.1 M Tris, 5 mM EDTA, pH ~8). Incubate at 60°C for 1 hour.
4. Add 100 µL 81 mM Iodoacetamide in Guanidine solution (end concentration 20 mM). Alkylate in the dark, 37°C for ½ hour.
5. Spin down 14000 x g to remove liquid.
6. Wash filter two times with 200 µL 50 mM NH_4HCO_3, spin down at 14 000 x g in between.
7. Move the 10K filter to new clean tubes.
8. Add 100 µL of digestion solution (5ng/µL trypsin in 50 mM NH_4HCO_3-solution) on the filter. Incubate 37°C, overnight (15 hours).
9. Wash filter with 2 times with 150 µL 50 mM NH_4HCO_3. Spin down at 14 000 x g.

IDENTIFIED PROTEINS



SPECTRUM



From ResearchGate.net:

https://www.researchgate.net/search.Search.html?type=question&query=Hemoglobin%20removal&tabViewId=55b67b806307d9ce798b45a7&previous=researcher

Rosemary L Sparrow added an answer:

Do you know the method of Tsuchihashi M about haemoglobin removal? Do you know other simple and fast method to separate hemoglobin from supernatant?

Haemoglobin is a serious problem during purification and analysis of antioxidant enzymes from red blood cells. For this reason it could be necessary to remove Hb in order to obtain a clear supernatant.

Rosemary L Sparrow · Monash University (Australia)

Hi Arbace

The best method we have found to remove Hb is a product called HemogloBind by BiotechSupport Group: http://www.biotechsupportgroup.com/hemoglobind-hemoglobin-removal-and-capture

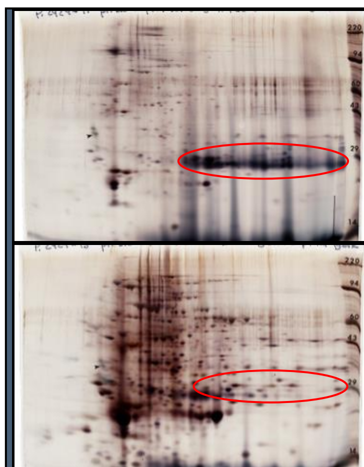
It is very easy to use and effective. I hope this is helpful. Rosemary

Hemoglobin Depletion and/or Enrichment

HemoVoid™

Hemoglobin Depletion For Erythrocyte Proteomics

- Hemoglobin voids in flow-through >98%
- depletion from heavily hemolyzed serum, whole blood and dried blood spot (DBS) card
- Low abundance protein and enzyme enrichment
- Consumable, cost-effective
- Mild elution maintains native structure with retained enzymatic, functional and bio-activities
- Species agnostic
- Compatible with LC-MS, activity-probe profiling and virtually all proteomic analyses



2DE Comparison. Red circles indicate the Hemoglobin subunits region. The HemoVoid™ eluate (bottom) has been severely depleted of Hemoglobin. The remainder of the red cell proteins are substantially enriched (visualized) and are better resolved in the HemoVoid™ eluate. Many more proteins are detectable after HemoVoid™ treatment with extensive proteome coverage across both dimensions.

Product	Size	Item No.
HemoVoid™	10 Preps	HVK-10
HemoVoid™	50 Preps	HVK-50
HemoVoid™	100 Preps	HVK-100

Based on 300 µl preps

HemoVoid™ LC-MS On-Bead

Hemoglobin depletion plus low abundance protein enrichment with optimized on-bead digestion for LC-MS erythrocyte & whole blood proteomics

- Seamless workflows, unique proteolytic efficiencies
- Label, label free & phospho- compatible
- See page 5 from our full catalog for more information and ordering

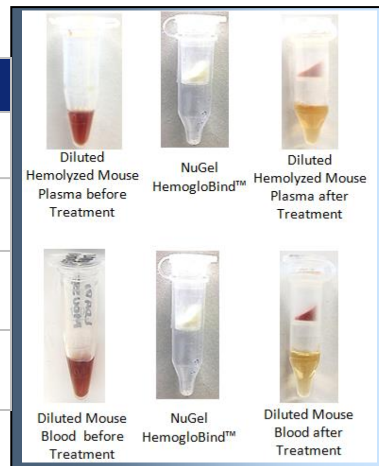
HemogloBind™ & NuGel™ HemogloBind™

Removes Hemoglobin Interference

- Highly specific for hemoglobin binding
- depletion from hemolyzed serum and whole blood
- applicable to hemoglobin variant analysis
- Functional integrity maintained with simple transfer to post-treatment interrogations
- Species and tissue agnostic
- supports biomarker tests

Product	Size	Item No.
HemogloBind™	15 ml	H0145 -15
HemogloBind™	50 ml	H0145 -50
NuGel™ HemogloBind™	25 Preps	NP-HO-T25
NuGel™ HemogloBind™	50 Preps	NP-HO-T50

1:1 v:v ratio HemogloBind™ suspension processes up to 10 mg/ml hemolyzed serum



Hemoglobin Removal Trial Kits

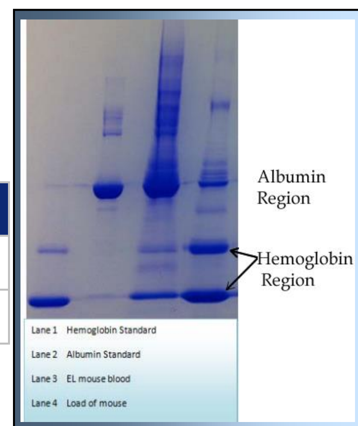
Product	Size	Item No.
HemoTrial™ Kit	5 ml HemogloBind™ + 5 Preps NuGel™ HemogloBind™ + 5 Preps HemoVoid™	HTK-05
HemogloBind™ Trial Kit	5 ml HemogloBind™ + 5 Preps NuGel™ HemogloBind™	HB145K -05

HemoVoid™ Blood Card Kit

The HemoVoid™ Blood Card kit substantially reduces hemoglobin interference from dried blood spot card protein analytes

Product	Size	Item No.
HemoVoid™ Blood Card	10 Preps	HVBC -10
HemoVoid™ Blood Card	50 Preps	HVBC -50

Based on 0.5" dried blood spot, ~ 15 µl whole blood



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