BIOTECH SUPPORT GROUP
Sample Prep that Matters

APPLICATION NOTE

The Influence of Sample Prep Bias on LC-MS Targeted Peptide Quantification in Serum Proteomics

MAY 6, 2021

Introduction

With advancements in LC-MS instrumentation and methods for targeted quantification, proteomic productivity is moving from protein identifications and Venn diagrams, to establishing quantitative differences between proteins in samples representing a challenge or disease state, vs. samples representing a normal or control state. For this, targeted LC-MS methods only measure peptides that meet both specific parent ion and specific daughter ion criteria corresponding to the target peptides of interest. The terms Selected Reaction Monitoring (SRM), Multiple Reaction Monitoring (MRM) and more recently Parallel Reaction Monitoring (PRM) all fundamentally exploit this label-free data acquisition method. Furthermore, targeted MS assays can be highly multiplexed, measuring up to several hundred peptides in a single acquisition. Its versatility extends to observation and measurements of functionally distinct proteoforms (i.e., active vs. inactive), post-translational modifications, along with splice and amino acid variants. Using exogenous chemically synthesized stable-isotope labeled (SIL) reference standards, even more precision and quantitative accuracy can be gained. Using SIL, peptide quantification is performed by comparison of the MS peak intensities from the light (endogenous) to the heavy (exogenous) peptides. For simplicity, we use the acronym MRM/SRM to collectively consider all these methods in this application report.

With the introduction of targeted analysis, sensitivity and technical variance in LC-MS/MS analysis has dramatically improved. Nevertheless, for serum/plasma samples, small biological variances remain hard to measure robustly. So validating small differences from discovery must translate to targeted quantification and that has become the main focus of BSG's enrichment/depletion product line.

In blood for example, the central Complement protein, C3 circulates at ≈1500 µg/ml, while Complement Factor D circulates at ≈3 µg/ml. To measure both in one analysis requires that the MS signal intensities at both ends of the spectrum be at least reasonably proportional to the real biological concentrations, which at times can be across > 4 log ion abundance signal. Thus low abundance proteins are foremost subject to signal to noise variance, making them barely detectable and often well beyond the range where signal intensity is proportional to concentration.

So, sample prep does matter. As an example, Kiseleva *et al* concluded that SRM measurements for particular proteins differed by orders of magnitude, using three protocols of sample preparation, as illustrated here.

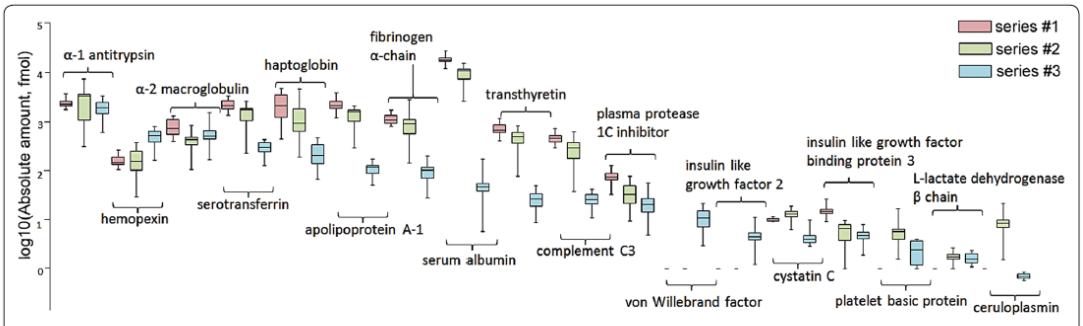


Fig. 2 *Box plot* for concentrations of FDA-approved proteins averaged across three technical runs and 19 plasma samples using three protocols of sample preparation (Series #1–3) From: Kiseleva *et al*. Clin Trans Med (2015) 4:32

By not relying on immuno-affinity for depletion, BSG has strived to address and service all these demands, to bring proteomics from discovery to the clinic.

The solution demands:

- ➢ Enrichment of biomarker proteins from low-abundance to mid-abundance to improve linearity between the measurable peptide ion signals and true protein abundances.
- ➢ BSG's enrichment products have proven to be robust, reproducible and quantitatively linear across >4x log of LC-MS/MS signal intensity data.
- ➢ Consumable products adaptable to high-throughput formats.
- ➢ BSG's On-bead (BASP™) digestion methods within AlbuVoid™ or HemoVoid™ simplified workflows, to help normalize digestion efficiencies for better protein quantification across multiple targets.
- ➢ A choice of enrichment/depletion strategies that can be used to optimize selection of surrogate peptides to report both total protein abundancies as well as functional proteoform features. BSG's Albumin & IgG removal products are characterized by the relative enrichment of particular sub-proteomes in the following table.
- ➢ Species agnostic, by not using immunoaffinity, all products are amenable for use with all species

	Apprx. plasma conc.	AlbuSorb™	AlbuSorb™ PLUS	AlbuVoid™ 4-hour BASP™ On-bead digest	AlbuVoid™ PLUS O/N BASP™ On-bead digest
Total Spectral Counts (SC)		**12203**	**14456**	**8969**	**23389**
Total Protein ID (≥2 SC)		**248**	**224**	**235**	**350**
% SC Albumin	**50-60%**	**11%**	**16%**	**1%**	**5%**
% SC Immuno-globulins	**10-20%**	**22%**	**12%**	**15%**	**6%**
% SC Apolipoproteins	**3-5%**	**6%**	**6%**	**11%**	**5%**
% SC Transport Proteins	**5-10%**	**20%**	**22%**	**17%**	**23%**
% SC Protease Inhibitors (i.e., Serpins)	**5-10%**	**24%**	**25%**	**12%**	**10%**
% SC Complement related	**5-10%**	**6%**	**7%**	**28%**	**31%**
% SC Coagulation/ Fibrinolysis	**3-5%**	**2%**	**2%**	**4%**	**5%**
% SC Other / Low Abundance	3-5%	**%9**	**10%**	**12%**	**15%**

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. Short time (2-4 hours) On-bead digestion (BASP™) with **AlbuVoid™** substantially reduces the influence of such Ig peptide features, as immunoglobulins are poor digesters. 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.

●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™ or AlbuSorb™ PLUS** is recommended.

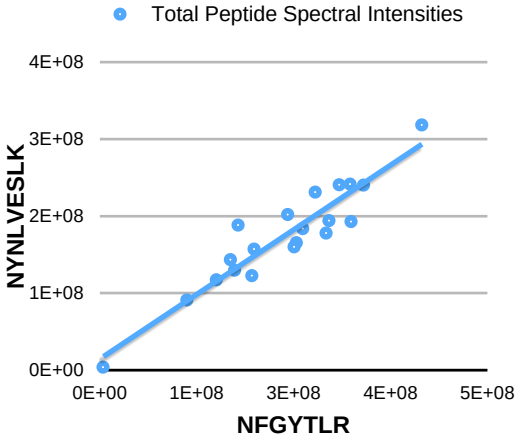
●The Complement sub-proteome is especially enriched by **AlbuVoid™**. Protease inhibitors, and notably the Serpin family are especially enriched by **AlbuSorb™**.

●The low abundance sub-proteome is enriched 4-5 fold with **all products**. Particular targets may be better enriched with a particular product, but this has to be determined empirically.

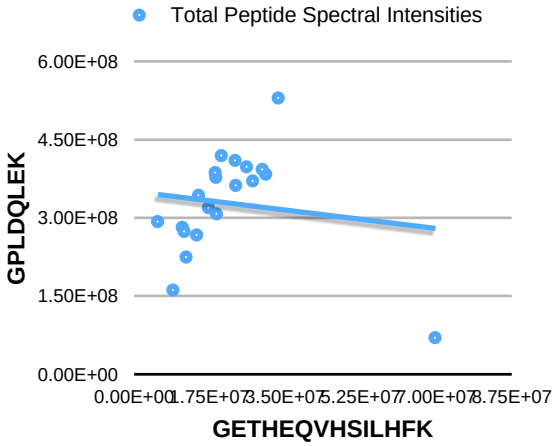
Digestion can be a large source of variation. A major source in serum/plasma is the high abundancy of immunoglobulins, a particularly poor digestion family of proteins, and heterogeneous in nature; it can distort digestion efficiency from sample to sample. Our recommendation is to:

Target assays to at least two peptides from the same protein to serve as an internal control for digestion efficiency. If the peptides align to the same abundance measurement, accounting for modest technical variance, an average value provides accuracy and assurance that all sources of sample prep variance are negligible. If the peptides do not align to the same measurement, then the test may be considered suspect.
For this strategy to work, the selection of peptides becomes an important consideration. Here are two examples from our serum knowledgebase, for the same protein in different samples; in this case sp|P05546|HEP2_HUMAN, Heparin Cofactor II, Serpin D1.

As a result the selection of internal standard SIL peptides should follow several criteria: (i) they should be proteotypic - that is, the amino acid sequence should be a unique identifier to the target protein(s); (ii) efficiently ionizable in order to provide good



In this example, the peptides align concordantly, tracking together to the same relative abundance.
GOOD SELECTION FOR TARGETS



In this example, the peptides do not align concordantly, consequently there is no assurance that either or both will track to relative abundance.
POOR SELECTION FOR TARGETS

detection by MS; and (iii) peptides with PTMs or that might be subjected to polymorphism should be avoided, unless as in some cases, with/without may be important target information. In all cases, however, the election of the proteotypic peptides to be used as standards in quantitative proteomics is approached in an empirical way, and makes use of those peptides that have been previously observed in discovery experiments. Once selected, the LC gradients, ionization properties, and isolation widths can be varied and optimized for signal resolution and acquisition time.

Here are some examples of BSG's sample prep products for discovery, targeted and clinical proteomic applications.

Jing, Lun, et al. "PROTEOMIC ANALYSIS IDENTIFIED LBP AND CD14 AS KEY PROTEINS IN BLOOD/BIPHASIC CALCIUM PHOSPHATE MICROPARTICLE INTERACTIONS." *Acta Biomaterialia* (2021).

Here, in a LC-MS/MS proteomic study, the authors compared the differentially expressed blood proteins (plasma and blood cell proteins) and the deregulated signaling pathways of osteogenic and fibrogenic blood biphasic calcium phosphate composites. The article describes use of **HemoVoid™** for depletion of Hemoglobin prior to LC-MS analysis, "each composite material or 4 blood clots were pooled into 3ml of cooled lysis buffer… They were let on ice for 30min … and centrifuged at 4°C, 8000g, 15 min. The supernatants were collected, and hemoglobin depleted using several **HemoVoid™** columns (Biotech Support Group). When indicated, albumin was also partially removed using **AlbuVoid™** depletion reagent kit (Biotech Support Group) following the manufacturer's instructions." From these enrichment steps, the investigators found respectively 80 and 92 proteins differentially expressed between blood clot and BCP 80-200 or BCP 200-500 blood composites. After **albumin depletion**, analysis of the significant deregulated proteins showed that 27 signaling pathways significantly changed in blood cells stimulated with BCP 80-200 particles compared to blood cells in blood clot, whereas only 6 of these pathways were significantly deregulated with BCP 200-500 particles. These data obtained after low abundance protein enrichment confirmed that the acute phase response protein, as well as LXR and. FXR pathways, were highly modulated in BCP blood composites but, conversely to what was observed without albumin depletion, these 3 pathways were more strongly altered by the BCP 80- 200 particles.

SCIENCE ADVANCES | RESEARCH ARTICLE

HEALTH AND MEDICINE

Lowering apolipoprotein CIII protects against high-fat diet–induced metabolic derangements

Ismael Valladolid-Acebes[1], Karin Åvall[1], Patricia Recio-López[1], Noah Moruzzi[1], Galyna Bryzgalova[1], Marie Björnholm[2], Anna Krook[3], Elena Fauste Alonso[1,4], Madelene Ericsson[5], Fredrik Landfors[5], Stefan K. Nilsson[5], Per-Olof Berggren[1,6,7,8,9], Lisa Juntti-Berggren[1]*

Increased levels of apolipoprotein CIII (apoCIII), result in obesity-related metabolic derangements. Using mice, the researchers investigated mechanistically whether lowering or preventing high-fat diet (HFD)– induced increase in apoCIII, protects against the detrimental metabolic consequences. For determination of circulating apoCIII, the article states, "plasma was albumin depleted using **AlbuSorb** according to the manufacturer's protocol (Biotech Support Group LLC) …"

Patented

Christer, M. A. L. M., et al. "Methods for the detection of autologous blood-doping." U.S. Patent Application No. 16/976,936.

The patent application relates to the identification of peptides, and the corresponding proteins, that can be used in methods for the detection of autologous blood doping. More specifically, the invention relates to methods comprising tryptic digestion of samples of isolated red blood cell (RBC), specifically isolated RBC cytosol, followed by peptide mapping using liquid chromatography tandem-mass spectroscopy (LC-MS/MS). The invention's description states "**Hemoglobin depletion** was performed using **HemoVoid** resin, buffers and filter tubes as described by the manufacturer (Biotech Support Group) using a standard bench-top centrifuge.". Upon the preferable depletion of hemoglobin, the methods enable detection of increased levels of certain peptides in samples from subjects that have been subjected to autologous blood doping, compared to samples from non-doped control subjects.

Springer

Klatt, Stephan, et al. "Optimizing red blood cell protein extraction for biomarker quantitation with mass spectrometry." Analytical and Bioanalytical Chemistry (2020): 1-14.

Red blood cells (RBCs) might serve as a reservoir for biomarker research as they are anuclear and lack the ability to synthesize proteins. Yet few biomarker assays have been conducted on RBCs because of their large dynamic range of proteins, high abundance of lipids, and the large amount of hemoglobin interference. In this study, the author's developed a semiquantitative mass spectrometry–based assay that targeted 144 proteins and compared the efficiency of urea, sodium deoxycholate, acetonitrile, and **HemoVoid™** as a sample prep step for the RBC proteome. The article describes the advantage of **HemoVoid™** in detection of low abundance proteins when comparing their amounts (in percent) between with the other four extraction conditions. With respect to Parkinson's Disease, the article states "For example, PRDX6 accounts for 0.4% of the total ion abundance after DOC (deoxycholate) extraction, whereas following HV (**HemoVoid™**) extraction, this increases to 8%, a 20-fold enrichment". The authors conclude that the **HemoVoid™** method significantly reduces the concentration of hemoglobin, resulting in an increased signal-to noise of the remaining proteins. This is especially useful for low abundance proteins and for those which might be close to the limit of detection without depletion.

RESEARCH TRENDS

Vialaret, Jerome & Kadi, Sarah & Tiers, Laurent & O Flynn, Robin & Lehmann, Sylvain & Hirtz, Christophe. (2018). Albumin depletion of human serum to improve quantitative clinical proteomics. Current Topics in Peptide & Protein Research 19. 53-62.

http://www.researchtrends.net/tia/abstract.asp?in=0&vn=19&tid=26&aid=6192&pub=2018&type=3

Proteins are essential for biomarker and disease discovery though the most abundant proteins often provide very limited clinically relevant data, as can be the case for albumin or immunoglobulins. In this work, the investigators focused on depleting albumin from human serum samples using an albumin depletion and low abundance protein enrichment kit – **AlbuVoid™**, which enabled the detection of several low-abundance proteins. By employing an optimized protocol, enriched proteins known as biomarkers for various diseases were identified. The article states "Protein capture was performed using **AlbuVoid™** Kit on serum samples… For the LCMS analysis, elution was made directly on the beads with denaturant solution (30 µL of 8 M urea / 20 mM DTT / 100 mM Tris pH 8.5) before tryptic digestion. In comparison, methods using antibodies needed at least one-half day more. The albumin depletion method allowed to save precious time. ". The authors concluded that the **AlbuVoid™** depletion method proved to be faster and more cost-effective than antibody based methods, and could be helpful for biomarker enrichment and detection in medical research.

This ebook describes how to address quantitative differences between proteomes in blood and serum. For this purpose, BSG's products and methods can help proteomic investigators explore unresolved inflammatory stimuli and new categorization strategies for selection of innate immunity biomarker proteins. Finally, the ebook describes how to improve information on the central regulators of the innate response - serine protease inhibitors.

https://www.biotechsupportgroup.com/v/vspfiles/templates/257/pdf/Categorization-of-Blood-Based-Biomarkers-v2.pdf

nature

Autophagy maintains tumour growth through circulating arginine

Poillet-Perez, Laura, et al. "Autophagy maintains tumour growth through circulating arginine." *Nature* (2018): 1.

Autophagy is a cellular mechanism which captures intracellular components and delivers them to lysosomes, where they are degraded and recycled, helping cells survive during times of starvation. One *in vivo* model to study autophagy is whole-body deletion of the essential autophagy gene *Atg7* in adult mice which causes a systemic

metabolic defect that manifests as starvation intolerance. In order to measure the systemic proteomic response of such deletion in this study, **AlbuVoid™** was chosen as one method to remove albumin and enrich the low abundance proteomes from serum. The article states "…**AlbuVoid** (Biotech Support Group) was used to deplete albumin…". This was done for the purpose of estimating differential protein abundances by LC-MS derived spectral counts. The serum proteomic investigation identified arginine-degrading enzyme arginase I (ARG1) in the circulation of *Atg7*-deficient hosts as one of several differentially identified proteins. Such evidence helped the authors' conclude that defective autophagy in the host leads to the release of ARG1 from the liver and the degradation of circulating arginine, which is essential for tumour growth; thus identifying a metabolic vulnerability of cancer.

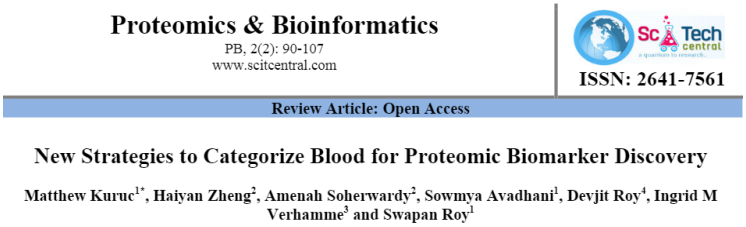
Whitepaper entitled "Stroma Liquid Biopsy™ - Blood-based biomarkers to monitor stromal conditioning in cancer." Published February, 2019. http://www.biotechsupportgroup.com/v/vspfiles/templates/257/pdf/StromaLiquidBiopsyWhitepaper022519.pdf

The whitepaper describes that tumors are more than simply a collection of immortalized cells as the supporting microenvironments or stroma also contributes to pathogenesis. Because of this, tumor characterization cannot be fully characterized solely through the analyses of the tumor cell genome – the current emphasis of liquid biopsy platforms.

The unique significance of the Stroma Liquid Biopsy™ pan-cancer profile is that dysregulation in blood was categorically intertwined with the most rudimentary needs of cancer: space, nutrients and immune evasion. Moreover, the changes within the 13 biomarker panel all occur within an interdependent network of cascading proteolytic events. Because proteolysis is irreversible, all species of life have evolved molecular regulatory systems to control aberrancies; the most distinguished is a protease inhibitory family of regulators known as SERPINs.

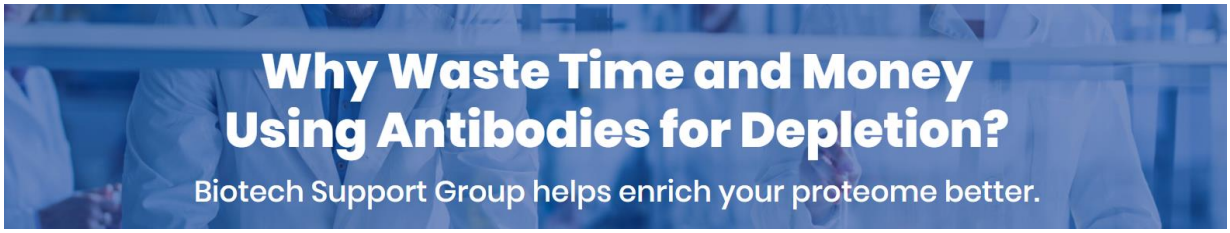
Zheng et al., "AlbuVoid™ Coupled to On-Bead Digestion - Tackling the Challenges of Serum Proteomics". J Proteomics Bioinform 2015, 8:9 DOI: 10.4172/0974-276X.1000373.

Using 2 different allotted digestion times - 4 hours, and overnight, each with a singular 3 hour gradient LC-MS run, between 400-500 total proteins were observed for both human and rat sera, with mostly overlapping but also with distinct sub-populations observable at each digest time. These results support that the described methods gain efficiencies over antibody depletion and in-solution digestion workflows, for both discovery and quantitative serum proteomic applications.



Many proteins are measurable in blood, making it a rich resource for biomarkers. In this review, we describe how chronic illness manifests itself in blood and how we might study innate immunity to understand mechanisms that can potentially translate into new biomarkers and therapeutic modalities.

We note that BSG's products have simply and efficiently reduced the complexity of the serum proteome allowing for cost-effective workflows, without the use of antibody-based depletion methods.
(4) (PDF) New Strategies to Categorize Blood for Proteomic Biomarker Discovery. Available from: https://www.researchgate.net/publication/341218896_New_Strategies_to_Categorize_Blood_for_Proteomic_Biomarker_Discovery [accessed May 10 2021].



Why Waste Time and Money Using Antibodies for Depletion?

Biotech Support Group helps enrich your proteome better.

Cost Effective & Efficient

Sample prep methods essential for expanding proteomic biomarkers into routine healthcare.

Consumable Research Products

Supporting the expanding installation of LC-MS instrument & computational infrastructure.

Knowledgebase of 1000+ Serum Proteins

Supports targeted & quantitative protein markers from serum/plasma.

Serves All Proteomic Analytical Platforms

Mass Spectrometry (LC-MS & MALDI),
Immunoassays, ELISAs & Western Blots,
1 & 2 DE,
Enzyme & Functional Assays

A Full Range of Albumin Removal Products

to Overcome the Challenges of Clinical Proteomics

AlbuVoid™

An Albumin Depletion reagent kit. Selectively voids Albumin, binds to and enriches low abundance serum proteome.

LEARN MORE

AlbuSorb™

An Albumin Depletion reagent kit. Selectively binds Albumin, not based on blue-dye or immuno-affinity.

LEARN MORE

AlbuVoid™ Plus

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

LEARN MORE

AlbuSorb™ Plus

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

LEARN MORE

Explore our other products for proteomic sample prep and matrix interference removal

Sample Prep Mass Spectrometry

- Proteome Enrichment
- Not based on immuno-affinity
- Bead Assisted Sample Prep (BASP™)

Products

- AlbuVoid™ LC-MS On-Bead
- HemoVoid™ LC-MS On-Bead
- Cleanascite™

VIEW ALL PRODUCTS

Hemoglobin Removal Kits

- Unique surface chemistries
- Depletes Hemoglobin 90-95%
- Species agnostic

Products

- HemoVoid™
- HemogloBind™
- HemoVoid™ LC-MS On-Bead

VIEW ALL PRODUCTS

Sample Prep - Proteomic Liquid Biopsy

- Urine
- Blood/Serum/Plasma
- Saliva/Sputum
- Synovial Fluid
- Broncheolavage
- Exosomes

VIEW ALL PRODUCTS

Specific Enrichment - Multiple Applications

- Kinases
- Glycoproteins
- Viruses and viral components

Products

- KinaSorb™
- NuGel™ PBA
- BindPro™

VIEW ALL PRODUCTS

About Biotech Support Group LLC
Converging with cultural and technological disruptions forthcoming in healthcare, Biotech Support Group develops methods for cost effective and efficient sample prep essential for these expanding markets. Following a tiered business strategy, the company continues its growth in the consumable research products area supporting the rapidly expanding installation of LC-MS instrument and computational infrastructure. For this market, key products include: AlbuVoid™ and AlbuSorb™ for albumin depletion, Cleanascite™ for lipid adsorption, and HemogloBind™ and HemoVoid™ for hemoglobin removal. From these innovations, the company has acquired knowledgebase and biomarker intellectual property assets that support discoveries of protein markers from blood, with special emphasis on early detection and personalized medical decisions for cancer patients. For more information, go to https://www.biotechsupportgroup.com/Default.asp

For business development,

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