

Path to Discovery

ProteoMiner™ Protein Enrichment Technology Goes Global

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Learn About How Experts in Proteomics Are Using ProteoMiner Kits

Hailed as the completion of a major scientific milestone, the sequencing of the human genome has in many respects marked the beginning of a new, more complex era of research. With roughly 25,000 human genes identified and the sequencing of many other types of plant and animal genomes either completed or under way, this research frontier requires not only identification of the estimated tens of thousands of proteins coded by these genes, but also determination of how gene expression or suppression correlates with the manifestation or prevention of disease. Because of alternative splicing events and posttranslational modifications, the highly complex proteome is more dynamic than the genome, and is therefore an additional rich source of potential biomarkers for clinical use. It is hoped that proteomics research will lead to the discovery of sensitive protein biomarkers that will aid in the diagnosis and prognostic assessment of disease, as well as in the development of more effective therapeutic interventions.



BIO-RAD

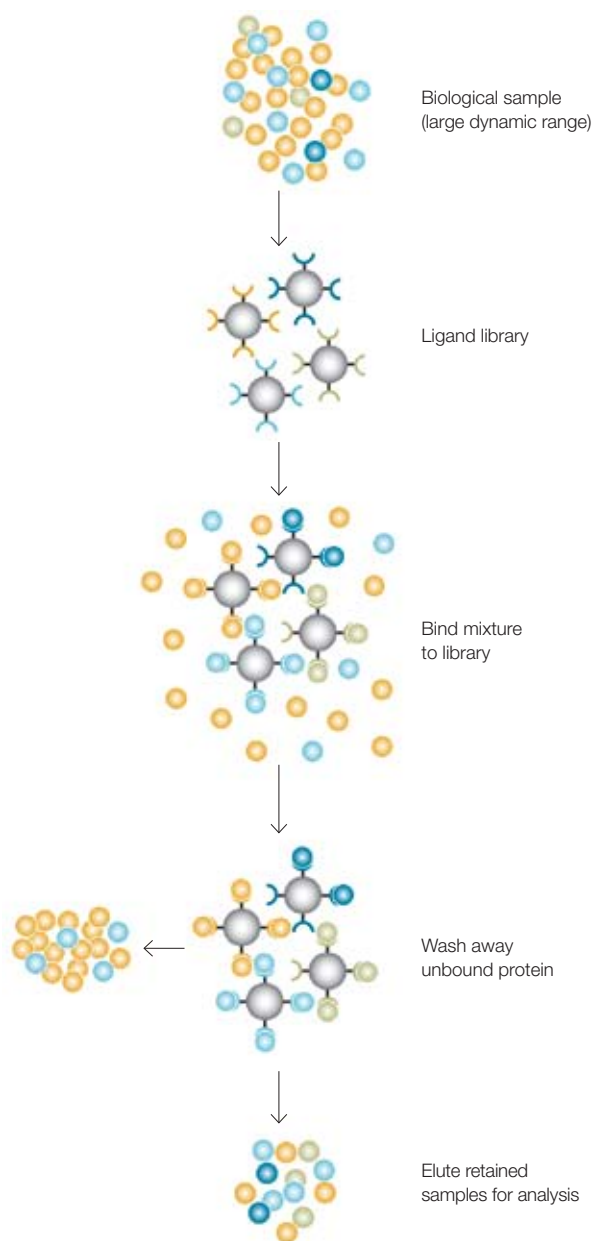


Fig. 1. ProteoMiner protein enrichment technology. Each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. When a complex protein sample is incubated with a bead library, protein components find their binding partners. Excess high-abundance proteins will not be captured on binding sites and will be depleted once the beads are washed. In contrast, low-abundance proteins will be concentrated on their specific affinity ligand. In this way, low-abundance proteins are enriched relative to the high-abundance proteins in the sample. No fraction is discarded in this approach, and proteins that might bind to high-abundance proteins like albumin are retained.

The task of identifying proteins related to disease has proved difficult because a relatively small number of high-abundance proteins in biological samples usually comprise a large percentage of total protein mass. For example, the proteins in human serum and plasma — the primary sample sources used in clinical proteomic studies — span a concentration range of 11 orders of magnitude. Furthermore, the 20 most abundant proteins represent 97–99% of the total protein mass (Anderson and Anderson 2002). When common analytical methods are used, these high-abundance proteins can inhibit detection of low-abundance proteins that may be related to physiological dysfunction.

ProteoMiner protein enrichment technology was developed to decrease levels of high-abundance proteins during the preparation of biological samples for proteomic analysis (Figure 1). This technology has been shown not only to render low-abundance proteins detectable, but also to increase their concentration, unmasking and enabling the discovery of hundreds of proteins. Results from studies using ProteoMiner protein enrichment technology indicate that the number of existing proteins has been significantly underestimated (Sennels et al. 2007). The capacity of this sample preparation tool to help unveil the proteome has led to its worldwide use in laboratories that are involved in the discovery of protein biomarkers for diseases.

ProteoMiner Protein Enrichment Technology

By decreasing high-abundance proteins and capturing low-abundance proteins, this novel sample preparation tool compresses the dynamic range of protein concentrations in complex biological samples, leading to an enrichment of medium- and low-abundance proteins. The technology utilizes a library of hexapeptides that are created through combinatorial synthesis, and bound to a chromatographic support. The result is a very large library of hexapeptide ligands that act as unique binders for proteins. The bead population has such diversity that a binding partner should exist for most, if not all, proteins in a sample. Each bead has an equivalent binding capacity; that is, the capacity for binding high- and low-abundance proteins in the sample is equal. Since there are a limited number of binding sites per protein, high-abundance proteins quickly reach saturation, while low-abundance proteins continue to bind. Those high-abundance proteins that reach saturation cease binding, and the unbound excess protein molecules are washed out (Figure 1). After elution, the medium- and low-abundance proteins are enriched while the concentration of high-abundance proteins is reduced. This allows detection and identification of low-abundance proteins that cannot be discovered using other methods (Figure 2).

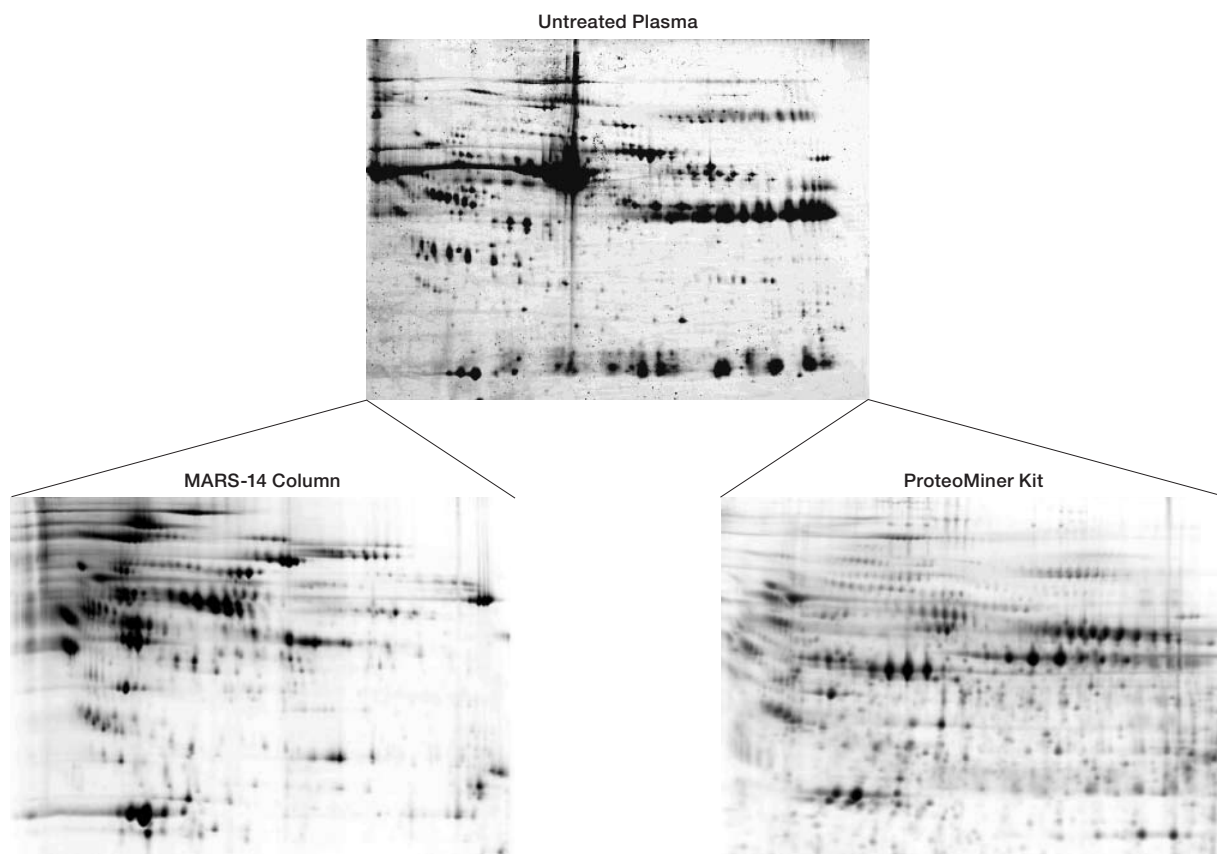


Fig. 2. Comparison of samples before and after treatment with the ProteoMiner protein enrichment kit and an immunodepletion product. Crude plasma along with plasma processed with either the ProteoMiner kit or an Agilent MARS-14 column were analyzed by 2-D electrophoresis. Top, untreated plasma sample; bottom right, ProteoMiner system-treated sample; bottom left, MARS-14 column-treated sample. Substantially more proteins were detected after treatment with the ProteoMiner kit. Data provided by AstraZeneca.

Prior to the release of ProteoMiner protein enrichment kits, immunodepletion was the most commonly employed method for depleting high-abundance proteins. Immunodepletion utilizes immobilized antibodies to selectively remove a subset of high-abundance proteins. However, immunodepletion does nothing to enrich low-abundance proteins, and actually results in their dilution. Furthermore, the capacity of the immobilized antibodies is very low, which limits the amount of sample that can be added. Researchers must process their samples multiple times in the attempt to retrieve low-abundance proteins in detectable amounts. Another disadvantage to immunodepletion is that many proteins associated with high-abundance proteins are inadvertently removed during the depletion step.

In contrast to immunodepletion, large sample volumes can be processed by the ProteoMiner protein enrichment kit, thus increasing the opportunity for capture and subsequent detection of low-abundance proteins (Figure 2). This enrichment technology is applicable to different types of sample, and has been used successfully with serum, plasma, urine, CSF, bile, and other sample types. With regard to samples, immunodepletion is more restrictive because antibodies used for depletion must be designed for specific sample types and species. Another key advantage of the ProteoMiner kit is that samples are eluted in small volumes, therefore no additional concentration steps are required for most downstream applications.

Going Global

Dr Stefan Lehr
Düsseldorf, Germany

Discovering Biomarkers for Diabetes Diagnosis



For more than 30 years at the German Diabetes Center in Düsseldorf, Germany, the latest techniques and research initiatives have merged with patient care to help elucidate all facets of the disease. Research efforts across

various in-house laboratories share the same goals: to gain insights into the physiological mechanisms of the disease, to reduce diabetes-associated complications, and to study and improve patient treatment.

It was this focus on translational research, the movement of discoveries in basic research to application at the clinical level, that induced Dr Stefan Lehr to join the center's research staff after receiving his PhD in biochemistry 8 years ago. Lehr's graduate studies at the University of Cologne had focused on signal transduction and the phosphorylation of insulin receptors, and included identification of specific phosphorylation sites and their physiological role in downstream cellular functions. Experienced in and enthusiastic about using sophisticated laboratory techniques such as mass spectrometry, 2-D gel electrophoresis, and subcellular fractionation, and aware that type 2 diabetes is a disease of increasing prevalence, Lehr established a proteomics lab at the center upon completion of his graduate program.

Today, Lehr continues to use cutting-edge techniques to analyze protein patterns related to type 2 diabetes. One aspect of the proteome lab is analysis of human serum samples to gain insight into the molecular pathogenesis of the disease. One of the challenges Lehr has faced when using techniques

such as 2-D differential gel electrophoresis, however, is the interference of high-abundance proteins. "Often the big spots (high-abundance proteins) mask the little spots (specific proteins of interest). Traditional methods for uncovering low-abundance proteins of interest involve several steps, in which you sometimes lose proteins," explains Lehr.

It was this major challenge that prompted Lehr's interest in Bio-Rad's ProteoMiner protein enrichment technology. Since then, Lehr has begun to perform testing with human serum to determine the applicability of this technology to his lab's research. Initial tests with ProteoMiner beads determined that protein profiling with human serum worked according to product manual specifications. Lehr and his team then went on to design experiments now in progress to assess the reproducibility and variability of the technology, as well as its compatibility with methods used in downstream analysis. "The crucial issue in comparative protein profiling is reproducibility," emphasizes Lehr.

With validation now successfully completed, Lehr plans to incorporate ProteoMiner protein enrichment technology into his workflow as a one-step fractionation method for quantitating low-abundance proteins. Ultimately he hopes these efforts will lead to the identification of biomarkers for the early stages of type 2 diabetes. "Frequently, the disease is diagnosed years after onset. During that lengthy period, disease is manifested and time lapses without treatment," says Lehr. He hopes that diagnosing the disease as much as a decade earlier than is currently possible will help prevent complications associated with type 2 diabetes — for example, myocardial infarction and stroke — as well as significantly reduce the costs associated with treatment.

Dr Pier Giorgio Righetti — Combining Science With Imagination

Throughout an illustrious career that began in 1965 with a PhD in chemistry, Dr Pier Giorgio Righetti has been, as he states, "accused of contaminating science with imagination." Raised by a father who basked in the humanities but struggled to earn a living, Righetti made a deliberate decision toward science, turning his back on perhaps more natural inclinations toward literature and poetry — though not entirely. As he went on to become one of the forefathers of capillary and 2-D electrophoresis, the many papers he published along the way were sprinkled ("contaminated") with literary allusions and metaphors.

But perhaps a hypothesis can be made for imagination as a requirement for scientific discovery — and you only have to trace Righetti's role in the development of ProteoMiner system technology to support this supposition. In 2004, Righetti became aware of Egisto Boschetti's concept to use ligand libraries for affinity-based protein extraction treatments. Over the last few years, Righetti and Boschetti have worked together to further the understanding of the molecular mechanisms between libraries and protein extracts, as well as to imagine a number of applications based on the concept of reducing high-abundance while enriching low-abundance proteins in human fluid samples.

Since the proteomics twist on this technology was announced in Siena, Italy, in 2004, Righetti and Boschetti have gone on to publish numerous papers detailing its efficacy. Most recently, they conducted a very fruitful collaborative

research effort to uncover the minority proteome of the red blood cell (98% is hemoglobin; the remaining 2% has been difficult to identify). Published reports have estimated this 2% to be composed of between 91 (Kakhniashvili et al. 2004) and 252 (Pasini et al. 2006) unique proteins. Using ProteoMiner technology, Righetti and Boschetti were able to identify over 1,500 (Figure 3). In true Righetti style, a review discussing these results includes a lengthy prologue metaphorically correlating the search for gold in the proteome with the waves of 49ers migrating into California on a search for gold in the 1800s (Righetti and Boschetti 2008).

When asked to imagine the effect of ProteoMiner system technology, Righetti says that this "first major advancement in proteomics research since the immobilized pH gradient" should have a major impact on the field by enabling:

- Identification of biomarkers for diagnosing pathologies in sera, spinal fluid, saliva, and other human fluids
- Development of drug treatments for diseases based on protein variations
- Identification of previously undiscoverable soluble protein allergens in plant- and animal-based food sources

History has shown that science in general and proteomics in particular has only benefitted from things that Righetti has been able to imagine.

Dr Jules Westbrook
Dublin, Ireland

The Heart of the Matter



In 1997, proteomics was emerging as a field of study, and Dr Jules Westbrook was earning his bachelor's degree in medical biochemistry at Brunel University in London. Westbrook spent 9 months working with Dr Michael

Dunn at Harefield Hospital, a world-renowned heart and lung transplant center, and that was all it took for him to become convinced that the application of proteomics to cardiovascular disease and heart transplants would revolutionize the understanding and treatment of heart disease. After receiving his degree, Westbrook returned to work in Dunn's lab, joining the transplantation proteomics and neuroproteomics group at the Conway Institute Proteome Research Centre at University College Dublin in Ireland.

The group's main research goals are an increased understanding of the molecular processes involved in heart health and disease, and identification of biomarkers that can be used for diagnosis/prognosis or as therapeutic targets. The specific emphasis of the cardiovascular research program is on heart diseases that result in end-stage heart failure, because these are among the leading causes of morbidity and mortality. "For example," explains Westbrook, "we are very interested in dilated cardiomyopathy, or DCM for short, since this is a disease of the heart muscle. As the disease progresses, the heart fails."

The group is particularly concerned with understanding how protein expression is altered in DCM hearts and, by using proteomics techniques to compare normal and diseased hearts, identifying differentially expressed proteins. Human serum, plasma, and tissue samples are the primary source materials used in these projects. According to Westbrook, the

masking of low-abundance by high-abundance proteins is a well-known challenge to extracting meaningful proteomics information relating to a disease or biological process. "Of course, there are many methods available for removing the most abundant proteins from serum or plasma," says Westbrook, "but there is always the risk of losing proteins of potential interest through nonspecific binding interactions."

When Westbrook and his colleagues became aware of the ProteoMiner protein enrichment kit, they were eager to see whether the technology could be used reproducibly to reduce levels of high-abundance proteins without their exclusion (since these might well have significance in disease), yet allow the enrichment of low-abundance proteins. "So far," says Westbrook, "we've tested the kit using human plasma and serum. We're impressed by what we have seen when comparing gel images of untreated samples and samples processed using the ProteoMiner kit."

Results have clearly demonstrated areas of reduction and enrichment, and the reduced areas correspond to the locations where high-abundance proteins migrate. And reproducibility has been confirmed. "We've processed different aliquots of the same samples and produced the same protein profiles on 2-D gels, which is, of course, indicative that the same proteins are being retained to the same degree by the beads," explains Westbrook.

The group is currently following up on initial work by running replicates of treated and untreated human plasma for statistical analysis, and will be using mass spectrometry to attempt to identify the proteins comprising newly visualized spots from enriched and reduced areas. "Looking ahead," says Westbrook, "we think the kit will produce some interesting results when we analyze serum taken from patients with dilated cardiomyopathy. We are very keen to see whether there is enrichment of heart-specific proteins that we know are present."

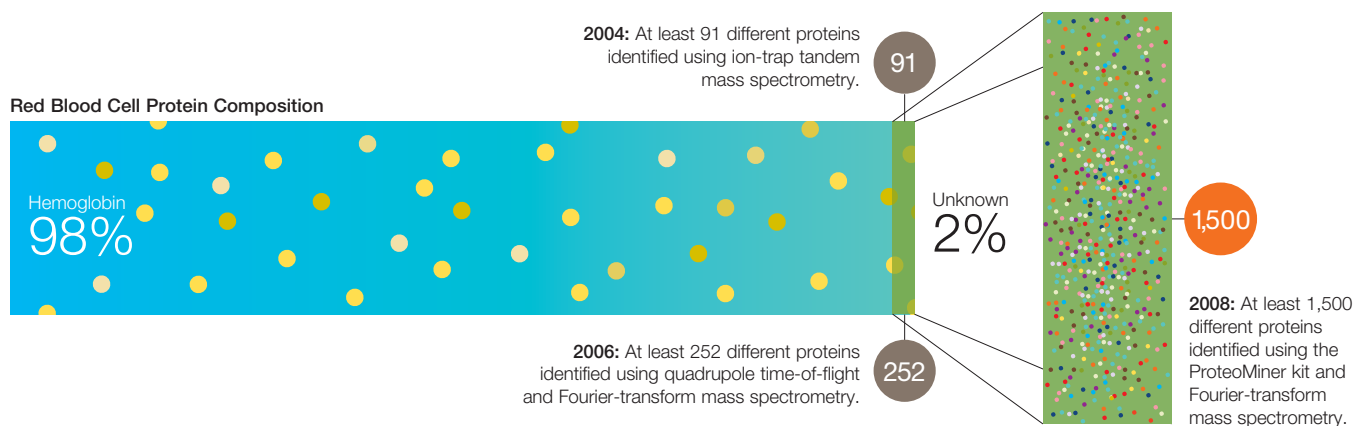


Fig 3. Approximately 98% of the protein composition of a red blood cell is hemoglobin, while the exact protein composition and make up of the remaining (bottom) 2% is unknown, because hemoglobin masks these low-abundance proteins. Analysis of the bottom 2% of proteins between 2004 and 2006 led to estimates of between 91 (Kakhniashvili et al. 2004) and 252 (Pasini et al. 2006) distinct proteins. Recent experiments by Righetti et al. using the ProteoMiner kit led to a revised number of at least 1,500 proteins, a 6-fold increase over previous reports.

Dr Ben Herbert
Sydney, Australia

Exploration Without Boundaries



Ignoring boundaries is not a new concept for Dr Ben Herbert, Director of the Proteomics Technology Centre of Expertise at the University of Technology, Sydney, Australia. In fact, it is a characteristic that can be traced

to the beginning of his career when, fresh out of polytechnic school, he was hired as a lab technician to test sausage content for New Zealand's food industry. This first job and level of education seemed enough for Herbert, until he was asked to help run tests in a study characterizing food proteins. This role sparked an interest in protein separation techniques and led to his pursuit, first of a university degree, and then a PhD in biochemistry. His ability to think outside the box led him initially to commercial ventures, but he was eventually drawn back to academia, where his current role allows him to combine independent thinking with directing scientific research.

Staff scientists are encouraged to ignore traditional boundaries and extend the limits of scientific possibility. "We don't really pay attention to instruction manuals," says Herbert. When Herbert was approached by Bio-Rad to test the ProteoMiner protein enrichment kit, initial tests were conducted using serum and plasma according to manual guidelines. "We do not do a lot of work with serum and plasma," explains Herbert, "so we just tested it and verified that it worked." But the success of these preliminary tests provoked the question, "What else can we use this for?"

A lab conducting research on paralysis ticks became the natural first choice. Found primarily on the eastern coast of Australia, a bite from this tick can cause paralysis and even death in dogs and cats. "In this research, you have to deal with a huge amount of blood from engorged ticks," Herbert explains. Researchers decided to grind up engorged ticks, place the homogenate onto ProteoMiner beads, and work through the abundant animal proteins present in host blood

cells to try to isolate proteins specific to the engorged tick. The ultimate goal is to analyze and identify differences in the protein content of the tick when feeding versus fasting, thereby identifying the proteins causing paralysis.

In another lab at the center, researchers are studying *Cryptococcus gattii*, a potentially fatal lung-infecting fungus. Studies are in progress to compare proteins found in the fungus in its native state with those extracted from infected lung, liver, and kidney samples. Without first processing these tissue samples with ProteoMiner protein enrichment technology, it would be difficult, if not impossible to isolate the fungal proteins from the infected tissue samples. "With the ProteoMiner kit, we can make sure we are not just seeing the top set of animal proteins," says Herbert. "It allows us to remove the animal proteins from the infected organism to uncover those that cause infection."

Perhaps the Proteomics Technology Centre's most boundary-stretching work using ProteoMiner protein enrichment technology concerns the study of cell membrane proteins. Because these proteins are integral to filtering and pumping materials into and out of cells, Herbert believes their characterization will prove crucial to the development of drugs that can be targeted and delivered with precision. There are two major challenges in this area of research, however: membrane proteins are in very low abundance, and they are not as hydrophilic as other types of proteins. According to Herbert, the means for simultaneously analyzing the predicted large numbers of membrane proteins did not exist until ProteoMiner protein enrichment technology. So Herbert and colleagues used the ProteoMiner kit to develop experiments using organic solvents (for example, methanol and trifluoroethanol) to help solubilize and therefore allow the capture of low-abundance membrane proteins. His prediction is that 30% of the low-abundance proteins they are beginning to isolate will be proteins essential to cell membrane permeability.

Conclusions

ProteoMiner protein enrichment technology is being used in laboratories throughout the world to expand what is known about the proteome. It is hoped that the continual discovery of proteins that were previously undetectable because of the dominating presence of high-abundance proteins will lead to the discovery of biomarkers for many debilitating and deadly diseases.

References

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Dig Deeper!

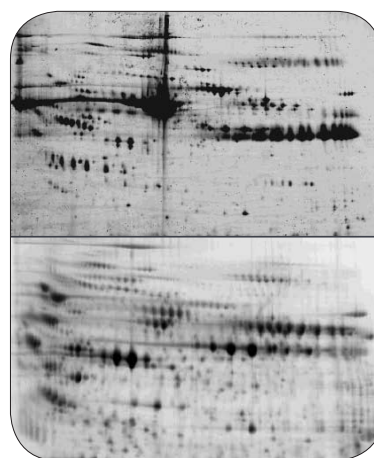
Uncover low-abundance proteins with Bio-Rad's new ProteoMiner protein enrichment kits.

ProteoMiner protein enrichment technology is a novel sample preparation tool for reducing the dynamic range of protein concentrations in complex biological samples. The presence of high-abundance proteins in biological samples (for example, albumin and IgG in serum or plasma) makes the detection of low-abundance proteins extremely challenging. ProteoMiner technology presents a method for overcoming this challenge.

- Decrease the amount of high-abundance proteins utilizing a combinatorial library of hexapeptides rather than immunodepletion to prevent co-depletion of low-abundance proteins
- Enrich and concentrate low-abundance proteins that cannot be detected through traditional methods

Whether you use one- or two-dimensional electrophoresis, chromatography, surface-enhanced laser desorption/ionization (SELDI), or another mass spectrometry technique, ProteoMiner kits enable enrichment and detection of low-abundance proteins.

Visit www.bio-rad.com/ad/proteominer/ for more information.



The ProteoMiner protein enrichment kit improves resolution and increases spot counts in two-dimensional gels. Top, untreated plasma; bottom, plasma sample treated with the ProteoMiner kit. Data provided by AstraZeneca.



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