Enrichment of Medium- and Low-abundance Proteins in Sample Types Using ProteoMiner™ Technology

Steve Freeby, John Walker, Aran Paulus, Kate Smith, Ning Liu, and Katrina Academia, Laboratory Separation Division, Bio-Rad Laboratories, Inc. 6000 James Watson Drive Hercules CA 94547 USA

Introduction

The field of Biomarker Discovery has traditionally been focused on serum and plasma, primarily due to their availability and ease of collection. Recently, there has been a trend toward beginning Biomarker Discovery projects with other sample types such as proximal tissues and fluids which are thought to contain the most disease-specific protein compostion. Furthermore, serum and plasma are challenging samples to analyze as the protein concentration range spans eleven orders of magnitude, and protein biomarkers are generally low-abundance proteins which are extremely difficult to detect in these highly complex samples. Working with less complex samples may alleviate these challenges. However, other biological samples present similar challenges with a small number of high-abundance proteins masking the low-abundance proteins of interest.

The high-abundance proteins in serum and plasma comprise over 98% of the protein mass, with the top two, albumin and IgG, comprising over 70% of it (Anderson and Anderson 2002). The dynamic range in other sample types, such as muscle tissue, bacteria, and cell lines also pose a challenge for low-abundance protein detection. In the proteome of muscle tissue, such as heart, myosin and actin constitute greater than 40% of the total protein population (Tajsharghi, 2008). In cell lines such as HeLa, actin constitutes greater than 25% of the protein population (Fountoulakis et al. 2004). In bacterial samples such as *E. coli*, there is no single high-abundance protein, but a set of medium-abundance ones which dominate the total protein population. An effective strategy to enrich for medium- and low-abundance proteins (from diverse sample types) to levels that allow for separation, detection, and mass spectroscopic identification is essential for increasing the number of candidate biomarkers.

Several methods have been developed to reduce the dynamic range of proteins in serum and plasma. One method is to remove the highest abundant proteins utilizing Cibacron Blue F3-GA and protein A affinity chromatography to remove albumin and IgG respectively. (Ahmed et al. 2003). This concept was expanded to immunodepletion techniques using specific antibodies to remove the top 6, 12, or 20 of the highest abundant proteins (Chromy et al. 2004). This approach works well but because it uses specific antibodies it has the disadvantage of being species specific, and it is restricted to serum and plasma samples.

As an alternative to immunodepletion, Bio-Rad Laboratories, Inc. has developed the ProteoMiner protein enrichment technology, which uses a diverse library of hexapeptides bound to a chromatographic support to bind all proteins in a complex mixture allowing reduction of high-abundance proteins and enrichment of medium and low-abundance proteins (Thulasiraman et al. 2005, Boschetti and Righetti 2008, Hartwig et al. 2009, Paulus et al. 2009, Righetti and Boschetti 2008). The hexapeptide bead library is highly complex and a binding partner should exist for every protein in a sample mixture. Following sample application, high-abundance proteins quickly saturate the binding partners for which they have highest affinity and excess is washed away, while low-abundance proteins are concentrated on their binding partners. The dynamic range of the protein concentration is reduced when the high abundance proteins saturate their ligands and the low abundance proteins bind to a sufficient number of ligands to allow for enrichment (Figure 1).

While the ProteoMiner technology was initially developed for biomarker applications with plasma and serum samples, it has the potential to be applicable to a wide range of sample types because the hexapeptide affinity is independent of sample source. The benefit of working with serum and plasma samples is that they have a high protein concentration (>50 mg/ml) with high solubility and require no pretreatment prior to processing. Other sample types have unique characteristics that include different dynamic ranges and solubility. In order for ProteoMiner to enrich proteins, the proteins must bind to the hexapeptides (through classical protein affinity interactions) and the high-abundance proteins must reach saturation. Therefore, the amount of extracted protein should be maximized while using buffer conditions that are compatible with the binding conditions of the ProteoMiner kits. Typical protein extraction buffers for 2-D gel

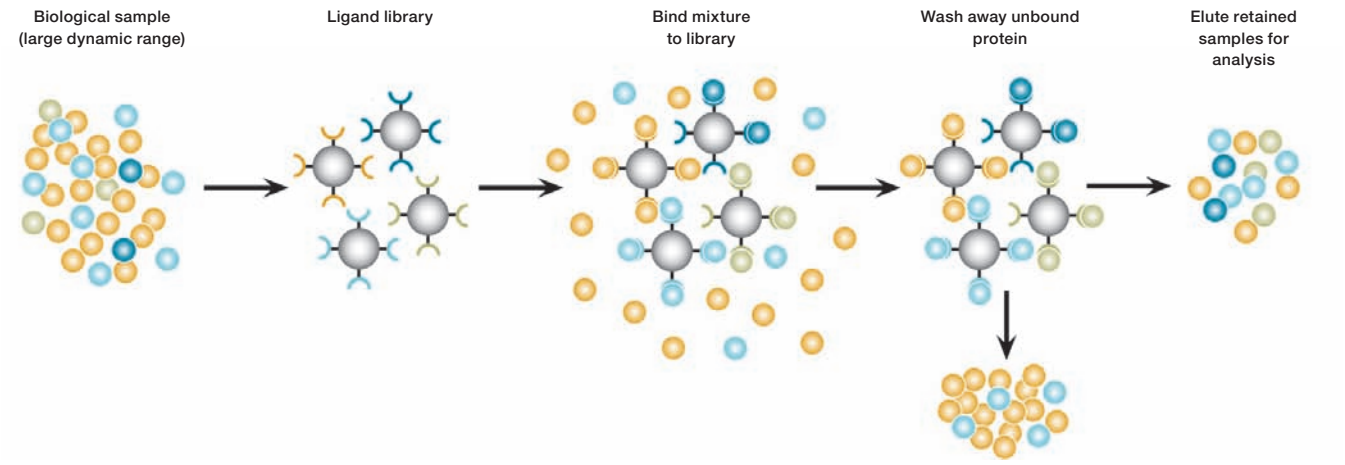


Fig. 1. Illustration of the mechanism of sample binding to ProteoMiner beads. ProteoMiner technology is based on a combinatorial library of hexapeptides bound to a chromatographic support. Each bead functions as a specific ligand. A complex sample is incubated with the ProteoMiner beads and proteins bind to the ligands to which they have the highest affinity. Unbound proteins consist primarily of high-abundance proteins and are removed during a wash step. Bound proteins are then eluted for analysis.

electrophoresis containing urea and CHAPS cannot be used with ProteoMiner beads as both urea and CHAPS denature the proteins and do not allow them to bind to the hexapeptides.

Alternatively, it is possible to solubilize the majority of proteins in 1% SDS during the extraction. Under these extraction conditions and following 10 fold dilution of the sample in 1 x PBS to reduce the SDS to 0.1%, protein binding to the ProteoMiner beads is still possible*.

This Tech Note discusses the use of ProteoMiner protein enrichment kits for the enrichment of low- and medium-abundance proteins and the depletion of high-abundance proteins in tissue (heart muscle), cell lines (HeLa cells), and bacterial (*E. coli*) samples, with detailed experimental parameters in a 2-D gel based proteomics workflow.

Methods

Protein Extraction – Heart Muscle, HeLa Cells and *E. coli*

Porcine heart was harvested and immediately perfused to remove excess blood from the tissue. The heart was placed on ice for transport and stored at –80°C. For protein extraction, 3 g of heart muscle tissue was diced into small, 1mm pieces in liquid nitrogen. A volume of 13 ml extraction buffer (1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.8, 2 mM PMSF, 1 mM EDTA and Complete Mini protease inhibitor cocktail tablets (Roche Applied Science) was added to the heart tissue. Samples were disrupted in a dounce tissue homogenizer (Wheaton) and sonicated on ice. Extracts were cleared by centrifugation (34,000 x g, 4°C, 30 min), and the resulting supernatants were stored at 4°C.

HeLa cells were harvested and placed into 7 ml of extraction buffer (same as heart extraction buffer above) and proteins were extracted using the same protocol as described above.

E. coli cells were grown to exponential phase in LB media at 37°C and collected by centrifugation (3,000 x g, 4°C, 5 min). A total of 2 g of cells were added to 3 ml of extraction buffer (same as heart extraction buffer above).

For each sample type, a control (untreated) sample was collected from the supernatant for subsequent 2-D analysis. For ProteoMiner bead treatment, 1 ml of the protein extract from each sample was diluted with 9 ml of PBS (1:10 dilution) prior to addition to a ProteoMiner column to lower the concentration of SDS to 0.1% which will not interfere with binding to the ProteoMiner beads.

ProteoMiner Bead Treatment

ProteoMiner treatment was according to the protocol supplied by Bio-Rad Laboratories, with the exception of sample load. It is recommended to use a 50 mg protein load with the large capacity kit, however, due to extraction yields this may not possible. In each example above refer to Table 1 for loading amount.

One- and Two-Dimensional Electrophoresis, Image Analysis, and Protein Identification

Proteins eluted from Proteominer beads were precipitated with the 2-D Cleanup kit (Bio-Rad) prior to 2-D electrophoresis to remove any ionic contaminants and dissolved in rehydration buffer (7 M Urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, 2 mM TBP and 0.2% Bio-Lyte® ampholyte 5–8). For 2-D gel electrophoresis, 100 µg of protein was loaded onto an 11 cm ReadyStrip™ IPG strip, pH 5-8 (Bio-Rad). Isoelectric focusing was performed using a PROTEAN® IEF Cell (Bio-Rad) at 8,000 V for 35,000 volt-hours. The IPG strip was transferred onto a Criterion™ Tris-HCl 8–16% gradient gel (Bio-Rad) and run for 1 hr at 200 V. Gels were stained with Flamingo™ fluorescent gel stain (Bio-Rad). The gels were imaged

* See considerations for processing samples using the ProteMiner protein enrichment kits, Tips and Technniques, BioRadiations 128, pg 29, 2009. Considerations to ensure compatibility include ensuring samples do not contain high level of ionic detergents such as SDS or NP40, the sample has a neutral pH (6–8), salt concentration is 50–150 mM and removing acidic polysaccharides, nucleic acids, lipids and polyphenols.

using the Molecular Imager® PharosFX™ system and the resultant gel images were analyzed with the PDQuest™ 2-D Analysis software (Bio-Rad). Selected spots were excised with the EXQuest™ spot cutter (Bio-Rad) and digested with trypsin prior to protein identification with mass spectrometry recorded on a Bruker AutoFlex II MALDI-TOF (Bruker Daltonics) instrument. FlexControl software was used for data acquisition and FlexAnalysis and BioTools software were used for data processing.

All samples were fractionated using the ProteoMiner protein enrichment kit. The controls and ProteoMiner fractions were analyzed by protein assay to evaluate the mass balance of the samples prior to and after fractionation (Table 1).

Table 1. Protein mass yield following ProteoMiner treatment.

Sample Type	Protein applied to column	Protein in Flow through	Protein eluted	Protein mass removed
Heart	26 mg	24.6 mg	704 µg	97.3%
HeLa cells	26 mg	23.9 mg	1.36 mg	94.8%
E. coli	20 mg	24.1 mg	764 µg	96.2%

In all sample sources, greater than 94% of the sample protein mass was removed (washed away as excess) using the ProteoMiner protein enrichment kit. The majority of the sample protein mass is present in the flow through (Table 1) or wash (not shown).

Results

ProteoMiner Treatment Reduced High-Abundance Proteins in All Sample Types Tested

Analysis of ProteoMiner bead-treated and untreated protein samples using 2-DGE revealed that the use of ProteoMiner beads increased the protein spot counts in all samples as compared to control untreated ones (Table 2). The protein spots that were significantly reduced by ProteoMiner treatment are circled in red in the control gels images (Figures 2–4). Since the high-abundance proteins were significantly reduced, more unique protein spots were visible.

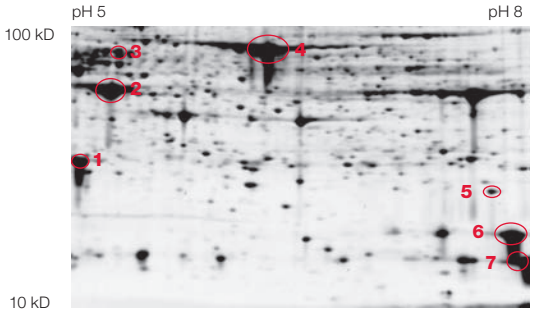
Table 2. Total spot count comparison between control (untreated) and ProteoMiner treated in different sample types.

Sample	Control Spot Count	ProteoMiner treated Spot Count	Percentage increase in spots detected
Heart	285	332	14
HeLa cells	315	435	27
E. coli	273	372	26

Heart muscle showed a reduction of myosin light chain (spot 1), actin (spot 2) and serum albumin (spot 4), which constitute greater than 40% of the total protein mass (Table 3). The presence of serum albumin is due to some blood remaining in the heart tissue even after perfusion. The ProteoMiner bead-treated protein gel showed an additional 47 unique protein spots as compared to the untreated sample (Figure 3). HeLa cells showed a significant depletion of tubulin (spot 1), and actin (spot 4) proteins, which constitute greater than 25% of the total protein mass (Table 4). This resulted in an additional 120 unique protein spots in the ProteoMiner bead-treated protein gel (Figure 4). Although *E. coli* samples do not have a very high-abundance proteins, a total of 8 higher abundance proteins were depleted (Table 5) resulting in 99 additional unique proteins in the ProteoMiner bead-treated protein gel (Figure 5).

Heart Tissue

A. Untreated



B. ProteoMiner bead-treated sample

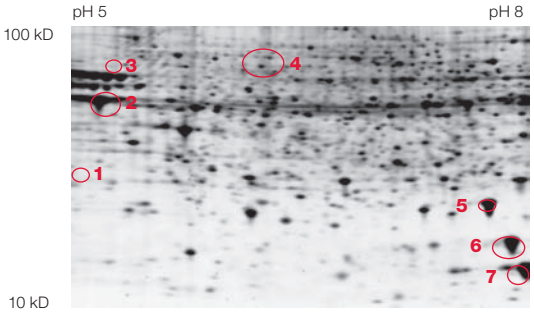


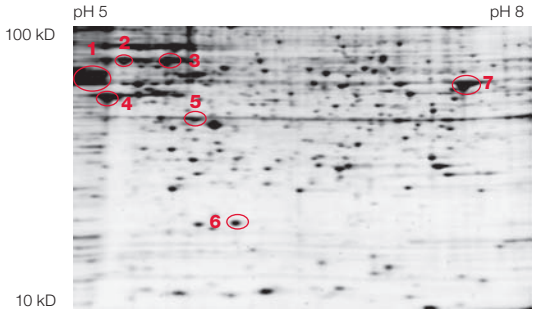
Figure 2. 2-D separation of heart protein extract. 100 µg of heart tissue protein extract was loaded on 11 cm pH 5–8 IPG strips for first-dimension separation and run on a 8–16% Criterion Tris-HCl gel for SDS-PAGE second-dimension separation. ○, indicate proteins that have been reduced in the ProteoMiner bead-treated sample (**B**) compared to untreated sample (**A**).

Table 3. High-abundance proteins from heart extract reduced by the ProteoMiner bead treatment. Protein spots were identified by MALDI-TOF mass spectrometry.

Spot #	Accession	Title	Mascot Score	MS Coverage	Protein MW	pI value
1	MLC1	Myosin light chain	63	61	~ 22,000	5.02
2	ACTH	Actin, gamma-enteric smooth muscle	83	31	42,249	5.20
3	DESM	Desmin	55	19	53,653	5.07
4	ALBU	Serum albumin precursor	87	21	71,643	6.07
5	CRYAB	Alpha crystallin B chain (Alpha B-crystallin)	46	37	20,116	6.92
6	MYG	Myoglobin	91	72	17,074	6.92
7	HBB	Hemoglobin subunit beta	87	81	16,212	7.96

HeLa Cells

A. Untreated



B. ProteoMiner bead-treated sample

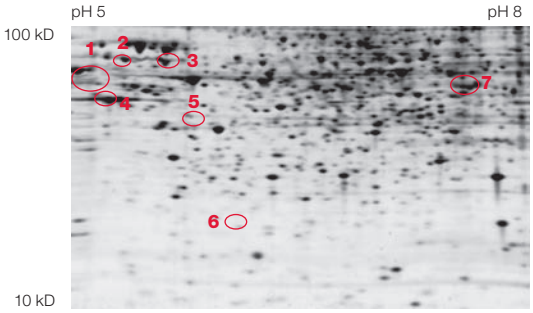


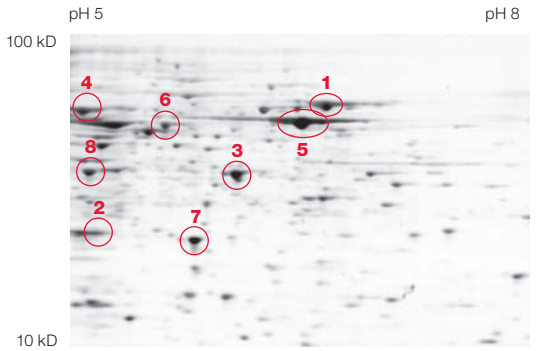
Figure 3. 2-D separation of HeLa cell protein extract. 100 µg of HeLa cell protein extract was loaded on 11 cm pH 5–8 IPG strips for first-dimension separation and run on a 8–16% Criterion Tris-HCl gel for SDS-PAGE second-dimension separation. ○, indicate proteins that have been reduced in the ProteoMiner bead-treated sample (**B**) compared to untreated sample (**A**).

Table 4. High-abundance proteins from HeLa cell extract reduced in the ProteoMiner bead-treated gel. Protein spots were identified by MALDI-TOF mass spectrometry.

Spot #	Accession	Title	Mascot Score	MS Coverage	Protein MW	pI value
1	TBA6	Tubulin α-6 chain (α-tubulin 6)	85	49	50,548	4.83
2	CH60	60 kD heat shock protein, mitochondrial precursor (Hsp60)	117	56	61,187	5.59
3	TCPE	T-complex protein 1 subunit ε (TCP-1-ε)	58	34	60,089	5.34
4	ACTG	Actin, cytoplasmic 2 (γ-actin)	109	77	42,108	5.20
5	G39	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	77	60	36,201	9.26
6	NDKA	Nucleoside diphosphate kinase A (EC 2.7.4.6) (NDP kinase A)	81	51	17,309	5.78
7	ENOA	α-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase)	103	49	47,481	7.71

E. coli Cells

A. Untreated



B. ProteoMiner bead-treated sample

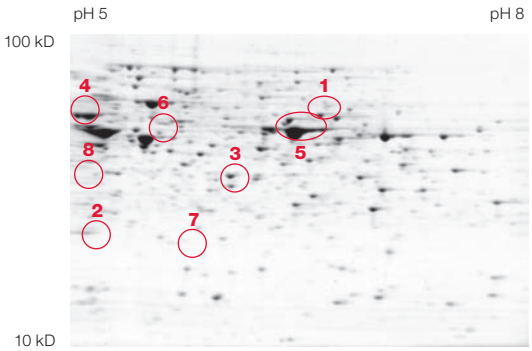


Figure 4. 2-D separation of *E. coli* protein extract. 100 µg of *E. coli* protein extract was loaded on 11 cm pH 5–8 IPG strips for first-dimension separation and run on a 8–16% Criterion Tris-HCl gel for SDS-PAGE second-dimension separation. ○, indicate proteins that have been reduced in the ProteoMiner bead-treated sample (**B**) compared to untreated sample (**A**).

Table 5. High-abundance proteins from *E.coli* extract significantly reduced by the ProteoMiner bead treatement. Protein spots were identified by MALDI-TOF mass spectrometry.

Spot #	Accession	Title	Mascot Score	MS Coverage	Protein MW	pI value
1	OPPA	Periplasmic oligopeptide-binding protein precursor	202	75	60,975	6.04
2	AHPC	Alkyl hydroperoxide reductase subunit C (EC 1.11.1.15) (Peroxiredoxin)	141	85	20,862	4.89
3	MDH	Malate dehydrogenase (EC 1.1.1.37)	140	70	32,488	5.50
4	ACEA	Isocitrate lyase (EC 4.1.3.1) (Isocitrase) (Isocitratase) (ICL)	101	67	47,777	5.03
5	TNAA	Tryptophanase (EC 4.1.99.1) (L-tryptophan indole-lyase) (Tnase)	155	80	53,155	5.83
6	EFTU	Elongation factor Tu (EF-Tu) (P-43)	119	71	43,457	5.18
7	SODF	Superoxide dismutase [Fe] (EC 1.15.1.1)	75	44	21,310	5.54
8	EFTS	Elongation factor Ts (EF-Ts)	101	46	30,518	5.08

Conclusions

The results presented here demonstrate that the ProteoMiner protein enrichment kit is an effective tool for the depletion of high-abundance proteins from protein extracts from a variety of tissues (mammalian tissues, cell line, and bacteria). However to ensure compatibility, it is necessary to use an extraction protocol that produces samples in a buffer that permits protein-protein interactions and consequently binding to the ProteoMiner beads. The use of ProteoMiner technology in a 2-D gel electrophoresis workflow increased the population of detectable proteins 15 to 30% for the samples used in this study, while reducing levels of high abundance proteins. The increase in the number of proteins visible in the gel improves the chances of detecting medium to low abundance proteins and effectively increasing the protein coverage in these samples. ProteoMiner provides a solution in biomarker discovery that is not limited by the sample types.

Acknowledgements

The authors thank Ben Herbert (University of Technology, Sydney, Australia) for sharing unpublished data, and Dr. Aldrin Gomes (University of California–Davis) for sample sources and assistance with this work.

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Information in this tech note was current as of the date of writing (2009) and not necessarily the date this version (rev A, 2010) was published.

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Bulletin 5916 Rev A US/EG *09-0942 0210 Sig 1109*