



**Jan 1 2006 (Vol. 26, No. 1)**

[✉ Email article to a colleague](#) [🖨 Printer friendly version](#)

## **Drug Discovery: Protein Profiling Poised to Make its Mark**

### **Understanding which Proteins are Expressed Under which Circumstances**

Angelo DePalma, Ph.D.

Protein profiling, an independent, emerging sub-specialty of proteomics, is poised to provide unprecedented insight into biological events. Protein profiling is defined here as the quantitative assessment of protein expression levels.

As profiling evolves, the term will increasingly refer to the study of multiple proteins, protein forms, or protein families, almost always comparing two different states (diseased vs. healthy, treated vs. untreated, experimental vs. control).

As this approach is perfected, protein expression profiling will make its mark in both high-brow and high-value research markets, including the elucidation of protein-protein interactions and signaling pathways, finding biomarkers for drug discovery and development, serum profiling to identify patient populations that respond to various treatments, and, eventually, medical diagnostics.

Where proteomics is the study of a proteome in a given circumstance, including how proteins interact and function, protein profiling refers more specifically to which proteins are expressed, under which circumstances and times, and in which tissues.

What stands in the way is the sheer complexity of the proteome: 30,000 genes coding for between ten and thirty times as many proteins, with concentration ranges varying by 10<sup>12</sup>. Traditionally, researchers dreaded this huge dynamic range. As we will see later, it is possible to exploit it to advantage.

### **Numerous Techniques**

Many high-throughput protein identification and characterization methods developed for proteomics have been applied to protein profiling. These include gel electrophoresis, mass spectrometry, and protein microarrays.

Unlike DNA sequencing, where capillary electrophoresis changed everything by allowing high-throughput sequencing, no equivalent exists for proteins. "We need multiple techniques to understand how proteins behave, and which are involved in various disease states," says Andrew Bertera, who heads marketing for gene and protein discovery at GE Healthcare ([www.gehealthcare.com](http://www.gehealthcare.com)).

Earlier this year, GE launched its ECL (enhanced chemiluminescence) Plex Western blotting kit, which improves quantitation and multiplexing by analyzing two different proteins on the same gel or membrane. In the 2-D area, more than 160 references cite protein profiling using GE's Ettan DIGE (difference in-gel electrophoresis) system, which is read through the Typhoon or the newly launched Ettan DIGE Imager.

DIGE also analyzes two protein samples within the same gel, eliminating inter-gel variability.

"Previously, one would have to run the samples on separate gels. With DIGE, we use three different dyes, including the pooled sample internal control, and mix the samples together," notes Bertera. Also in the Ettan line is a multidimensional LC system that interfaces to an MS.

As profiling efforts will rely on multiple techniques, so will each depend on the perfection of numerous underlying technologies. Protein microarrays are a case in point. Yuxin Wang, Ph.D senior scientist at ProteinOne ([www.proteinone.com](http://www.proteinone.com)), identified high-throughput antibody generation technologies, artificial molecular recognition surfaces of target proteins, phage-display libraries of antibody fragments, and proteome arrays containing full-length, functional proteins from a library of expression clones.

"I think the next step in protein profiling will be to combine the existing methods and take advantage of different technologies, through an approach known as multiplexed protein profiling," comments Dr. Wang.

"This will bring improved specificity and sensitivity, greater parallelism, and higher throughput, which is where protein profiling should be headed." For example, ProteinOne's Active Protein Array is designed for the multiplex detection and assessment of multiple human protein interactions with DNA, RNA, and/or ligands of interest.

### **Limitations of Gels**

Although 2-D gel electrophoresis will never be replaced in protein characterization studies, it suffers from a few drawbacks. Gels are time-consuming, imprecise, and their results are difficult to reproduce. Gels work well for soluble, high molecular weight proteins, but not so well for low molecular weight or relatively insoluble species.

Bands on gels often contain several proteins, and great skill is required to assure uniform measurement across gels. "What you think is the major constituent may actually be quite a few proteins," notes Tim Riley, Ph.D., vp for proteomics business development at Waters ([www.waters.com](http://www.waters.com)).

On the plus side, 2-D gels offer a window to visualizing a thousand proteins or more in one analysis and are easily interfaced to mass spectrometry (MS). "But there is a lot of work associated with processing gel information," Dr. Riley adds.

To varying degrees, MS provides the same advantages for profiling experiments as for proteomics in general: high sensitivity and accuracy, mass and sequence information that is difficult to obtain otherwise, and reasonably high throughput.

For differential expression measurements, MS experiments are either isotopically label based or label free. In labeling experiments, the experimental and control proteins are generated and tagged independently, one with a "heavy" <sup>13</sup>C-hydrocarbon chain (or other stable isotope) and the other with a lighter version of the same label (typically with fewer <sup>13</sup>C atoms). The two samples are then combined, digested, and analyzed using LC-MS or LC-MALDI MS/MS. This technique labels at the protein level.

A related method uses <sup>18</sup>O during tryptic digestion, labeling all amino acids in either the control or experimental sample. However, in this technique the samples must be digested separately, resulting in a loss of internal control over digestion efficiency.

Waters supports both labeling and label-free methods, but lately has made great strides in the latter. Label-free strategies require balancing protein concentrations between control and experimental samples, but avoid the need for expensive derivatization reagents and the attendant complex chemical manipulations.

Coupled with MS, label-free protein profiling provides a reasonably precise mass analysis and relative

abundance for each peptide. Results show 1030% variability, about the same as with isotopic labeling. Waters' proprietary label-free technique applies normalization through an internal standard to arrive at relative intensity values.

Waters' flagship profiling offering is the Protein Expression System, which incorporates the Q-ToF Premier mass spectrometer and Waters' nanoAcuity UPLC separation system, plus standards, training, and standard methods for achieving good reproducibility in quantitative sample handling and analysis.

Waters' proprietary data acquisition technique for label-free experiments uses alternating high- and low-collision energy modes to acquire accurate mass values: the low-energy data for quantitative comparison, and the high-energy plus low-energy values for qualitative identification of peptides and proteins.

### **Dynamic Range is your Friend**

In medical diagnostics, the benefits of profiling several proteins versus a single biomarker is that together, multiple markers can provide close to 100% sensitivity and selectivity, compared with 30% to 50% for single markers.

"In biology having just one difference between disease and normal states is unlikely," says Mary F. Lopez, Ph.D., strategic collaborations leader at PerkinElmer ([www.perkinelmer.com](http://www.perkinelmer.com)).

A seminal article, by Petricoin and Liotta, appearing in *The Lancet* in 2000, was perhaps the first report demonstrating that profiling based on mass spectrometry (MS) could serve as a disease diagnostic. What was revolutionary about this work was the idea that high-abundance proteins can assist, rather than interfere with, proteomic analysis.

Carrier proteins in serum or blood, normally removed to reduce background noise, bind to lower-concentration biomarkers shed from tumors and other tissues.

"The best way to find these biomarkers is to capture the carriers and see what is bound to them," says Dr. Lopez, who described the original Petricoin and Liotta work as a "paradigm shift" despite the fact that the methods they used in 2000 were quite crude. Recently, the two researchers validated their idea by demonstrating spectral profiling and purification of ovarian cancer biomarkers captured through high-abundance carrier molecules.

However, protein profiling will not enter "prime-time" medical diagnostics markets without a significant boost in its ability to pick out protein markers at very low concentrations. "A high-resolution platform will provide confidence and statistical significance," says Dr. Lopez.

PerkinElmer demonstrated, in a recent paper on Alzheimer's disease markers, a technique for identifying biomarkers by capturing albumin on a 96-well plate, selectively eluting the markers, and analyzing them by MALDI MS. Selective elution uses a proprietary solvent that knocks the biomarker off albumin, which is retained on the plate.

A good deal of profiling employs protein (or antibody) arrays, the protein counterparts of successful gene arrays. Protneomix ([www.protneomix.com](http://www.protneomix.com)) specializes in protein arrays (especially antibody arrays) for drug discovery based on three core competencies.

The firm's proprietary, cell-free in vitro protein expression expands the numbers and types of available, "array-able" molecules to include cytotoxic and prokaryotic proteins. This synthetic capability can generate purified, tagged proteins from any organism with a sequenced genome. Through its antibody array manufacturing, Protneomix provides custom arrays on 3-D plane supports without chemical modification of targets. Finally, for protein interaction experiments, the company offers labeling and detection methodology operating at attomole (10<sup>-18</sup>) levels for immobilized proteins and antibodies.

Protneomix' technology platform allows comparison of expression levels for "many proteins" in a single binding assay (for example pre- and post-treated cells), relative toxicity evaluation, and biomarker discovery for cancer and other diseases.

"Our technology has replaced many thousands of Western blots," says Vehary Sakanyan, Ph.D., professor at the University of Nantes and a principal with Protneomix. "The benefit of very low sample consumption is especially relevant with biopsy material."

One application of antibody arrays is analyzing post-translational protein modifications, which cannot be done using DNA arrays. Here, antibodies are generated against proteins possessing specific phosphorylation patterns.

Antibody arrays are only as good as the antibodies they contain, but even the best exhibit cross-reactivity, which limits their reliability. To cut down on this phenomenon, Dr. Sakanyan suggests using only the highest-quality antibodies for spotting, depleting high-concentration proteins beforehand, and perhaps analyzing proteins from only one location in the cell.

"One might also consider replacing full-length antibodies with antibody fragments which retain high affinity and specificity, but with lower cross-reactivity," he adds.

Protein arrays have led to orders of magnitude more answers per experiment, says Brett Stillman, Ph.D., manager for microarray technologies at Whatman ([www.whatman.com](http://www.whatman.com)).

"Protein arrays also have the potential to replace more and more conventional assay technologies," he adds. Arrays, particularly antibody arrays, facilitate multiplex capture and are about as efficient as running individual ELISA assays, especially when sample volumes are limited and precious. Dr. Stillman likes the ability of protein arrays to monitor protein-protein interaction, calling them "more powerful" than co-immunoprecipitation.

Whatman's protein profiling product line includes the FAST slides, found in array products manufactured by other companies. More than 80 scientific papers cite FAST in their methods section.

Whatman's Serum Biomarker Chip, based on single antibody capture with direct fluorescent labeling of serum samples, profiles 120 of the most common serum biomarkers. The company also offers custom protein array and sample processing services in the above formats and others.

In first quarter 2006 Whatman plans to release a protein array diagnostic for the European market that will measure auto-antibodies associated with different collagenosis and vasculitis-related autoimmune diseases.

TeleChem-ArrayIt ([www.arrayit.com](http://www.arrayit.com)) has provided tools for microarray DNA analysis since 1996, and for the last few years has offered protein array products plus a line of microcontact printing devices for protein microarray manufacture.

ArrayIt's protein array tools are used by more than 3,000 research groups worldwide, including many academics who were responsible for developing methods based on these tools. The precision NanoPrint Microarray system (with 60 and 210 slide capacity) and the NanoPrint Protein Edition Microarrayer are high-throughput instruments for the manufacture of protein microarrays. ArrayIt offers the SpotBot microarrayer for personal, low-throughput array manufacture.

### **Protein Stabilization**

Proteins were once thought to be too delicate for long-term storage on microarrays, but that notion has been refuted. Arrays are printed on high-quality glass substrates using specialized printing buffers that stabilize proteins on the surface and attached to the glass covalently through appropriate surface chemistries. For example, arrays that employ ArrayIt's SuperAldehyde chemistry enjoy a shelf life of at

least one year on par with the stability of DNA microarrays.

ArrayIt will soon ship a new scanning architecture for microarrays based on a combination of charge-coupled device (CCD) and complimentary metal oxide semiconductor (CMOS) detection. What's unique about this system, called SpotLight, is the use of specific fluorophore excitation combined with CCD detection.

Traditionally, CCD detectors employ a white light source and filter sets to read fluorophores of interest. Here, instead of white light, fluorophores are excited specifically at the wavelengths they were designed for. "This dramatically reduced the cost of instruments without sacrificing sensitivity and dynamic range," says Todd Martinsky, co-founder and vp.

Post-translational modifications (PTMs; glycosylations, acetylations, phosphorylation, and others) dictate such protein properties as shape, function, reactivity, and how the body disposes of them.

Nutricognia ([www.nutricognia.com](http://www.nutricognia.com)) analyzes these patterns, and how processing affects them, in food proteins. "The same protein with two different glycosylation patterns could wind up in the liver or kidney," observes Ofer Markman, Ph.D., CSO. Food manufacturers pursuing health claims must be as cognizant of PTMs as pharmaceutical researchers.

Nutricognia's CarboDeep biochip analysis technology profiles milk proteins and produces a fingerprint of the glycan composition within a batch. The technology behind CarboDeep was licensed from Procognia ([www.procognia.com](http://www.procognia.com)), which applies it to drug development.

To Anke Cassing, Ph.D., associated director at Qiagen ([www.qiagen.com](http://www.qiagen.com)), multiplexing of ELISAs and MS techniques and quantification are the most notable trends in protein profiling.

Qiagen's MSXpress Protein Chip kits provide sensitive high-throughput analysis of proteins using MALDI-MS. Qiagen claims up to 100-fold improvement in sensitivity compared with conventional targets for identifying low-abundance species.

MSXpress 192 Protein Chips contain 192 precisely defined sinapinic acid matrix spots, which concentrate the sample protein within the spot volume. After the sample dries, it is crystallized and presented to the sample stage of a MALDI MS.

### **Adding Significance to Data**

Many companies offer reagent kits for profiling. Among these are Invitrogen, DNAMicroarray, Affinity Labeling Technologies, Promega, Schleicher & Schuell BioScience, and many others. Many of these firms have contributed to the science of protein profiling as well as delivering products.

Privately held Kreatech Biotechnology ([www.kreatech.com](http://www.kreatech.com)) specializes in sample preparation, labeling, and detection for protein characterization. The company's business segments include FISH (fluorescent in situ hybridization) probes, labeling kits, and sample preparation for DNA microarrays based on the company's Universal Linkage System (ULS).

ULS exploits the stable coordinative binding properties of platinum complexes to nucleic acids and proteins. ULS reagents consist of a platinum complex, a detectable molecule, and a chemical group displaced through reaction with the nitrogen and sulphur atoms of methionine, cysteine, and histidine on target proteins.

According to Roel Schaapveld, Ph.D., vp for corporate development, binding to both residues provides thorough proteome labeling. Kreatech is positioning ULS as a protein profiling tool for serum or cell lysate systems that traditionally suffer from high complexity and broad protein concentration dynamic range. "Scientists who use 2-D gels and MS for protein profiling during drug discovery are constantly looking for additional methods to add significance to their data," says Dr. Schaapveld. "The problem, which DNA researchers do not face, is the 10 log concentration scale from albumin down to rare

cytokines. No single technology can cover this entire scale of concentrations."