

# Biomarkers for Disease Identification

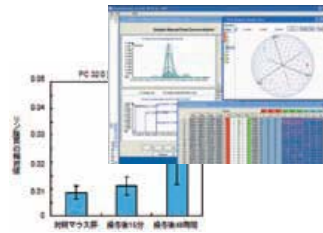
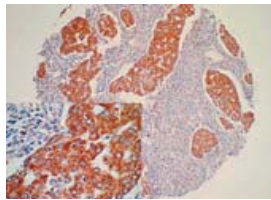
Shimadzu protein instruments open new avenues to speed biomarkers to the clinic

**Discovery**

**Verification**

**Validation**

**Application**



Is a patient really sick? What medicine is necessary? In what dosage? Is the patient responding to it? Doctors customarily answer these questions based on a variety of symptoms. But many of the symptoms used today, because of their subjective description and uncertain relationship to the disease state, are misleading.

Scientists are rushing to find new 'biomarkers' — biological molecules and physiological characteristics — that are more closely linked to the underlying causes of health or disease. Their discoveries are set to transform the practice of medicine by giving doctors a more objective and quantifiable basis for clinical decision-making. The proteins found in various tissues, the structure or concentration of which varies with disease progression, offer the most promising leads.

The discovery of such biomarkers, however, which must be plucked from tens of thousands of proteins that fill our cells, presents a challenge. If not identified with precision and validated in large patient groups, they could do more harm than good. New technology is needed. Fortunately it is here. Biomedical scientists, like the three introduced below, are using this new technology to address the challenge and fulfill the promise of biomarkers. Their instruments of choice come from Kyoto-based Shimadzu Corporation, a multibillion dollar enterprise with manufacturing bases in six countries.

## Separating the Bad from the Good

Daniel W. Chan Ph.D., DABCC, FACB

Professor of Pathology, Oncology, Radiology & Urology Director, Clinical Chemistry Division & Center for Biomarker Discovery Co-Director, Pathology Core Laboratories, Department of Pathology  
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In September 2009, the US Food and Drug Administration (FDA) gave the nod to Vermillion's ovarian cancer diagnostic test, OVA 1. It was the first blood test the agency cleared for estimating the risk of ovarian cancer. Based on five biomarkers (transthyretin, apolipoprotein A-1, beta2-microglobulin, transferrin, and cancer antigen 125), the test uses an algorithm to produce a numerical score indicating the likelihood of malignancy. It was the first of its kind — a protein-based in vitro diagnostic multivariant index assay — approved by the FDA.

The test had a long history. The tight link between the five biomarkers and disease was first demonstrated by the research group of Daniel W. Chan, a professor of pathology, oncology, radiology and urology at Johns Hopkins Medical Institutions<sup>1</sup>. In 2004, after the technology had been licensed from the university, a clinical trial with 600 patients found that it greatly improved sensitivity compared to the previous test, which assayed only cancer antigen

125. Further successful clinical work led to FDA clearance. "It is a great example of what we want to do — find biomarkers, validate them and then translate them to the clinic," says Chan.

As the potential of biomarkers comes to be further understood and appreciated, more such success is sure to come. Chan directs the Center for Biomarker Discovery at the university hospital. There he has assembled a team that includes specialists in cancer biology, mass spectroscopy technology and clinical diagnosis, as well as an electrical engineer who develops statistical bioinformatics tools.

The major targets of the group's studies are glycan modifications — the addition or removal of sugar molecules — to proteins. 'Glycosylation' is often up-regulated or down-regulated in cancer, so the amount of glycoproteins is a good indication of the presence of a tumor. But quantity alone is not enough to tell whether that tumor is benign or malignant. For that, other characteristics of the glycoproteins must be studied. For example, glycoproteins rich in sialic acid are known to help cancerous cells enter the blood stream and spread cancer, offering a potentially more accurate diagnostic tool that could enhance early detection of deadly cancers and at the same time reduce unnecessary surgeries. "It's a good marker of cancerous growth," says Chan.

Mass spectrometers are used to analyze the proteins in bodily samples. But conventional mass spectrometers often cannot distinguish proteins that share certain characteristics but differ in others that are crucial to understanding their function. To get that kind of resolution, a two-stage test is needed. Chan found success with the Shimadzu AXIMA QIT, a mass spectrometer that combines a quadrupole ion trap (QIT) and high-performance reflectron time-of-flight analyzer.

The Shimadzu AXIMA QIT "provides high mass accuracy and resolution in the peaks," says Chan. In addition to high precision and reliability, it enables a sample to be analyzed sequentially, with each fragment providing more minute detail, whether that be glycan modifications, sialic acid content or other characteristics. "It's a unique capability of this Shimadzu instrument. For complicated things with small differences in structure, it is the best," says Chan.

This hair-splitting precision will be important for detecting other kinds of cancer, says Chan. For example, tests for prostate specific antigen (PSA) could reveal not only the presence and amount of glycoproteins but also differentiate them into specific types. The Shimadzu AXIMA QIT technology could potentially improve the effectiveness of diagnostic tests such as the OVA1 test, says Chan.

With an improved version of the Shimadzu AXIMA QIT, the Resonance, on the way to his laboratory, Chan is excited. "The better the tests we come up with, the more we can help patients," he says.

## Structural Integrity

Samir M. Hanash

Program Head, Molecular Diagnostics, Fred Hutchinson Cancer Research Center

Samir M. Hanash of the Fred Hutchinson Cancer Research Center has perhaps the largest set of plasma proteins around — nearly 8,000. “That covers about one-third of the human genome,” he says. “It’s a phenomenal set of data adding up to many terabytes.” Armed with this resource, he will be taking on some of the most common and lethal disorders known to humans: heart, lung, colon, pancreas, breast and ovarian cancer, as well as heart disease and stroke.



This huge collection promises great benefits but it also poses a huge challenge in terms of analyzing the structure of the different proteins. “Quantity is not enough. We need to go deeper,” he says.

Hanash also relies on cutting-edge mass spectrometry-based analyses of glycan modifications. He says that one shortcoming of conventional mass spectrometry is that it usually requires cleavage of the glycan followed by separate analyses of the glycan and the deglycosylated protein. It is therefore impossible to see the critical glycan structure as it is in a mixture of proteins.

Hanash has been tackling this problem with Shimadzu’s next-generation mass spectrometer, the Shimadzu MALDI Digital Ion Trap (DIT). Hanash says his respect for Shimadzu has grown, especially because of the work of Nobel laureate Koichi Tanaka. “We appreciate what he has done, the resolution of the instruments, the ability to fly large proteins into the instrument, and the soft ware for analysis.”

Intact proteins are labeled with isotopes, fractionated with high-performance liquid chromatography, and then analyzed using the MALDI DIT. With its wide mass range, the MALDI DIT can analyze both the peptide and oligosaccharide components simultaneously. The instrument uses a square-wave electronic signal — the resemblance of which to a 0 or 1 digital signal gives the device its name — to trap the ion used to measure the molecule’s mass-to-charge ratio.

Through this multi-stage process, Hanash can tell not only which proteins are increasing or decreasing in concentration, he can also get precise information about their structures. “The first screen tells us which type of receptor is in circulation. But then we want to determine the exact structure of that type. With the Shimadzu device, we can zoom in on specific structures of the proteins,” he says.

As an illustration of the power of this new method, Hanash’s team analyzed a set of glycoproteins in the plasma that are known to differ in concentration between normal individuals and lung cancer patients. They examined 41 protein groups, including CPI, CPI, C4BPB, DNAH3 and UBR1. The study allowed Hanash’s group to note how the concentration of the proteins and how their glycan structure changed with physiological changes resulting from the disease. The result is a group of well-characterized and promising biomarkers. Hanash says there are three factors involved: the type of chemistry used to label the protein, the type of hardware, and the type of soft ware used to interrogate data. “You need all three and Shimadzu has them. We can do it all with Shimadzu.”

## Proteins, Individuals and Families/What's in a Name?

Dr. Fred E. Regnier

J.H. Law Distinguished Professor, Department of Chemistry  
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Fred E. Regnier is fighting a similar battle to sort through nature's complicated mixtures of proteins. His weapon is liquid chromatography (LC). Unfortunately, conventional LC only works with mixtures of 100 to 200 proteins. "When more than a thousand proteins are involved, which is oft en the case, you can't resolve them," says Regnier, the J. H. Law Distinguished Professor in Purdue University's chemistry department.

But if you take each of a 100 peaks and then separate those into 100 components in a 'second dimension', a process known as orthogonal analysis, then individual proteins can be singled out from a mixture of thousands. "This is the kind of resolution the scientific world needs now," says Regnier. Shimadzu's two-dimensional (2D) LC instrument is uniquely capable of fulfilling that need.

In Regnier's study on breast cancer, the first dimension is an affinity test that selects for molecules with lectin, a possible indication of cancer. With that, he narrowed 30,000 proteins down to 30. Shimadzu's 2D-LC instrument further homes in on the culprits using another property, such as size. Using this 2D analysis, Regnier's group found 15 proteins that are strongly associated with metastasis in breast cancer.

Regnier says the technique helps place blame where blame is due. In one dimension, you might find a family of proteins at fault. In two dimensions, you find that only one member of that group is linked to the disease. "All the members might have a similar feature and therefore be grouped together. But it's like members of a family — just because they have the same last name doesn't mean they are all the same. It would be like punishing a whole family for something that one individual did wrong," says Regnier.

The success could transform the way proteins are studied, beginning with the evaluation of antibodies. Antibodies might be assumed to bind a certain protein, but in actuality, they might be binding one or all of a family of similar proteins. If these proteins function differently, the distinction will become crucial. Regnier even used Shimadzu's 2D-LC device to discover some faulty antibodies. One such antibody was supposed to bind transferrin. It didn't. 2D-LC analysis showed why: the antibody was already saturated with transferrin. "You'd never know that with any other method except using the Shimadzu instrument." Regnier thinks regulatory authorities should start requiring people to use an instrument like Shimadzu's when validating antibodies.

Shimadzu's device has other advantages. Its automation makes possible experiments with picoliter amounts of sample that might, if transferred manually, dry up. "This is particularly important in clinical analysis," says Regnier. The Shimadzu instrument is also flexible. "You can assemble it in many ways. But when you get it together, it looks like it's made to be a single integrated unit. It's so compact," says Regnier.

Given the ease of use, it will bring biomarkers to the fore of medical care for a range of disorders. Regnier also hunts biomarkers for diabetes and heart disease. Such 'oxidative stress' diseases result from a modification of a protein's carbonyl group. "We can single out those molecules that are oxidized and even tell in which organ the oxidative stress occurred," says Regnier. That specificity helps with diagnosis: oxidative stress in the substantia nigra suggests Parkinson's disease; in the cerebrum, Alzheimer's disease; in the arteries, atherosclerosis.

In a study of ten 'normal' volunteers, four already had elevated levels of oxidized proteins in their arteries. "We can see indications of heart disease 20 years before they will face the risk of a heart attack," says Regnier. He thinks such diagnostics could be ready for the clinic within five years.

In another common test for PSA, useful in diagnosing prostate cancer, antibodies for PSA are used. But it turns out PSA is actually comprised of more than 50 different molecules. Most likely only a small fraction of those are related to cancer. "The Shimadzu device will let you see only the form related to disease. It will be the new standard in clinical diagnostics," says Regnier.