Biomarkers make their mark on current research and drug development trends

Biomarkers continue to become increasingly relevant in research and healthcare applications, as evidenced by the global market for products involved in their identification, validation, and use – estimated at $8.3 billion in 2007 and projected to increase to $15 billion in 2010[1]. The accelerating pace of activity in this area is further underlined by a cursory review of the publication space, where the number of relevant scientific articles generated annually has doubled from 20,000 to 40,000 over the past decade **(Figure 1)**.

By Stéphane Parent and Dr Gregory Cosentino

The NIH defines biomarker broadly as "a characteristic objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention"[2]. As such, this term can describe many common assays, physiological measurements, and imaging protocols that speak not only to biochemists, pharmacologists and toxicologists, but also primary healthcare providers. Narrowing the context to drug discovery, biomarkers serve a wide variety of applications from target validation through drug candidate development and clinical trial assessment. Both gene expression and protein analyte profiling have been successfully utilised in this regard, however the proteome offers advantages over the transcriptome with respect to the number of relevant variations in protein synthesis, turnover, localisation and post-translational modifications that associate to normal biology and disease states. Furthermore, protein analyte targets are often easily accessible through various biological fluids including serum, saliva, urine or cerebrospinal fluid.

The notion of a useful biomarker implies sensitivity and specificity to the biological state being considered, and must be supported by epidemiological evidence that links it to a clinical outcome. Validated biomarkers can be used as surrogate endpoints to substitute for a clinical end point in drug development, which then allows for early assessment of the drug benefit/efficacy. Moreover, selected biomarkers can be monitored in preclinical studies using cellular or animal models. For these applications to be useful, however, it is critical that the candidate biomarkers be rigorously qualified through a defined discovery and validation process that includes not only a deep understanding of the physiological relevance of the marker, but also experimental confirmation of the precision, robustness and limitations of its use for the intended purpose. Collaborations are essential to this effort, and a number of diverse stakeholders have now established mechanisms to harmonise approaches in

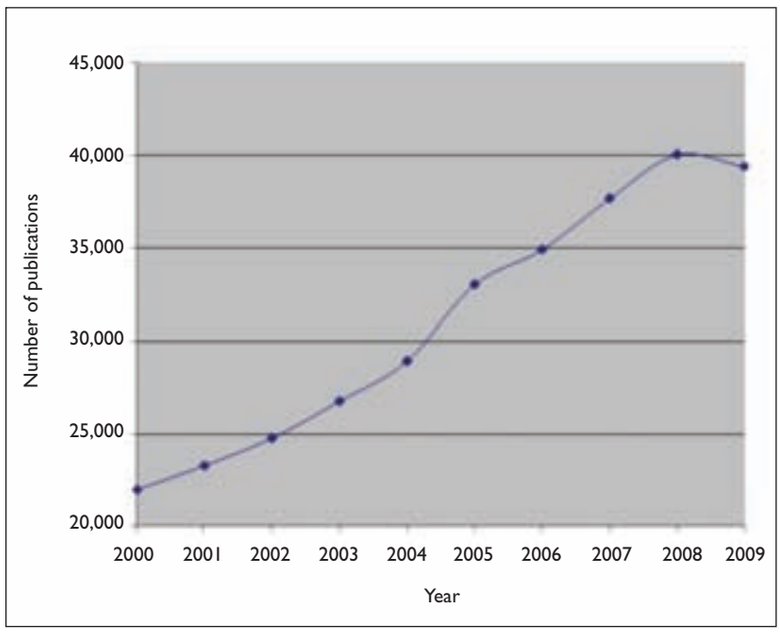

Figure 1
PubMed citations referencing biomarker as a key word between 2000-2009

order to drive the utility of biomarkers as efficiently as possible into research and clinical applications. A significant example of this is the Biomarkers Consortium, which was formally established in 2006 and brings together the resources of the NIH, medical and academic communities with the private sector represented by pharmaceutical, biotechnology, diagnostic and instrumentation companies. Such open-source precompetitive collaborations, funded by national or international programmes such as EU-FP6 in Europe or PhRMA in the USA, offer both challenges and significant opportunities, as reviewed recently following completion of the first Biomarkers Consortium project demonstrating potential utility of adiponectin as predictor of metabolic responses to PPAR agonists in diabetic patients[3,4]. Not surprisingly, important lessons learned from this inaugural study include the need for establishing common standards and definitions of successful biomarker qualification in an open data-sharing environment.

Application areas for biomarkers

Biomarker identification and validation has been applied to a wide variety of therapeutic areas including neurological disease, metabolic disorders, and immune dysregulation, but perhaps the most predominant field of application lies in oncology. As reviewed by Marrer and Dieterle[5], prognostic biomarkers are presently available to guide oncologists in formulating optimal treatment plans for their patients, with the two most cited examples being commercial kits for stratification of breast tumours based on the trancriptional profile of 70 genes (MammaPrint® Assay, Agendia BV) or 21 genes (Oncotype DX® Assay, Genomic Health Inc), respectively. These assays allow for clinical categorisation of patients for risk of disease recurrence, and therefore helps establish the need for follow-on treatment. The application of targeted cancer therapies fall under the rubric of predictive biomarkers, which address the potential response or insensitivity of the tumour to a particular therapy. In this case, companion diagnostic tests must be co-developed with the drug through clinical trials in order to not only demonstrate drug efficacy but also validity of the predictive test. One of the best known examples of a predictive marker is the Her2/neu diagnostic test for treatment of Her2 positive metastatic breast cancer with Herceptin.

A wide variety of accumulated studies have been published in the evaluation of potential diagnostic biomarkers for cancer, with varying degrees of success. Recently, an integrated systems biology approach was advanced by Kulasingam et al[6] in order to identify promising ovarian cancer markers. This strategy included proteome and transcriptome comparisons of tissue, serum, proximal fluids, cancer cell lines and animal models from a variety of sources. This group short-listed 33 candidate proteins common to three datasets, and two proteins which were overexpressed in four of the datasets studied. Both of these proteins were known to be associated with invasive ovarian cancer, and thus served to support the integrated approach to biomarker identification.

The application of biomarkers to improved pharmacodynamic and safety testing for the pre-clinical/clinical phase of drug development is also an important application area. This has already been well established through the clinical use of the microarray-based AmpliChip® CYP450 test (Roche Diagnostics Corp) which analyses patient genotypes for variant alleles of cytochrome P450 (CYP) genes, CYP2D6 and CYP2C19, which correlate with different phenotypic effects on drug metabolism. Recently, the first comprehensive set of studies supporting seven renal biomarkers for measuring drug toxicity were published by the Predictive Safety Testing Consortium (PSTC), representing a collaboration of scientists from 17 pharmaceutical/biotechnology companies, five academic institutions, and the FDA/EMEA[7]. One of the key principles applied to this effort was the adoption of well-defined criteria for analytical assay validation as outlined by the NIH Chemical Genomics Center and guidances contained in the

FDA Bioanalytical Method Validation for Industry. Assays were rigidly validated based on sensitivity, specificity, robustness, accuracy, precision, linearity, working range and analyte stability. Furthermore, these parameters were defined in the context of the biological matrix and potential interference effects from urinary components were assessed.

The development of additional toxicity biomarkers for other major target organs has also been described (reviewed by Marrer and Dieterle[8]). Hepatotoxicity is a major clinical challenge and a common cause of adverse events in drug development. While serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) continue to be accepted measures of liver injury, combination with other leakage biomarkers may improve sensitivity and specificity for this application. For example, glutathione-S-transferase is an early inducible detoxification enzyme in liver, and serum levels of this enzyme are a useful marker of organ histopathology. However, significant variation in baseline liver expression levels of this enzyme may limit establishment of a universal threshold for clinical application. Cardiac troponin I and T are established leakage markers for damage to the heart muscle, however NT-proBNP, the N-terminal cleavage product generated as a result B-type natriuretic peptide processing, may offer advantages such as improved stability and ease of measurement as a biomarker for ischaemic heart disease and ventricular dysfunction.

Measurement of protein biomarkers

Genome-wide analytical technologies continue to be applied to identify potential biomarkers of relevance to pathobiology and therapeutic intervention. Over the past decade, gene expression microarray technology has been well established and offers the ability to measure the transcriptome under a variety of biological conditions at either the single gene level or collectively in multiple genes[9]. Proteome analysis offers the additional advantage to evaluate changes at the protein level that may be overlooked by transcriptomics. High-throughput

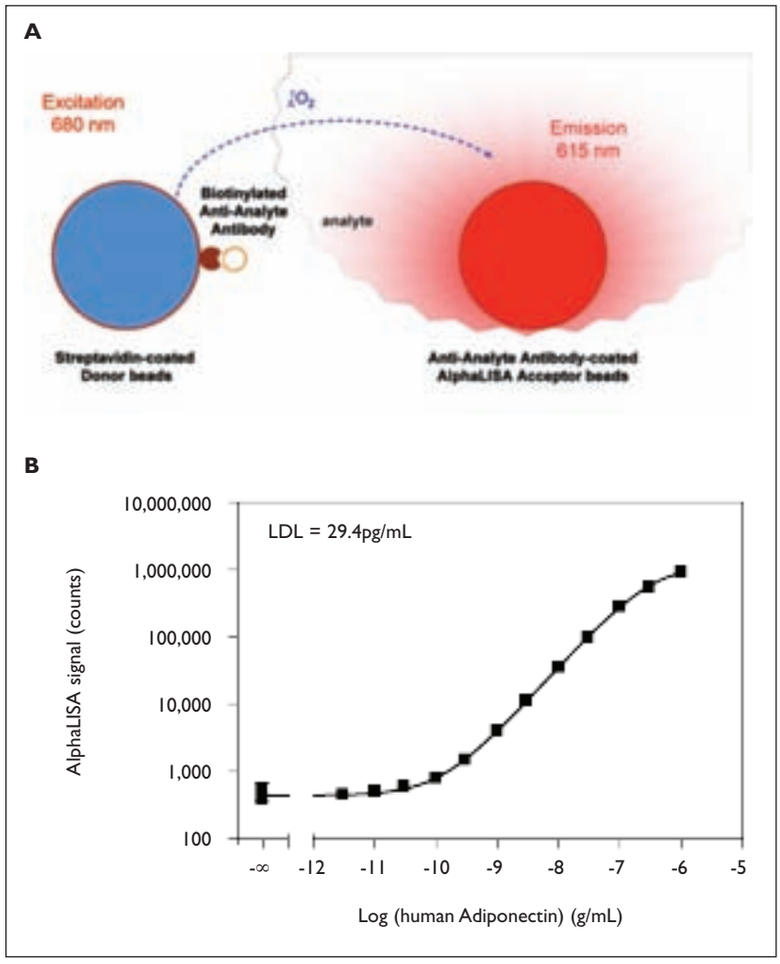

Figure 2
A) Generic assay configuration of an AlphaLISA assay for easy quantification of biomarkers. B) Standard curve for quantification of adiponectin obtained using AlphaLISA, a non-wash assay. The assay can detect as little as 29pg/mL of total adiponectin in serum

methodologies combined with adequate data handling and analysis can lead to the discovery of interesting unbiased biomarkers in either biological fluids[10] or in cell-based models[11]. Whether biomarkers are identified using unbiased strategies or selected based on *a priori* insights of signalling pathways, it is subsequently critical to develop rapid and accurate assay protocols that will allow their measurement on multiple samples in a broad range of matrices using proven, easy and reliable techniques.

ELISA and other antibody-based approaches

Sandwich ELISA is the gold standard in the quantification of protein biomarkers for research application. It is an easy and sensitive method relying on the use of two specific antibodies to the protein of interest. A microplate-bound capture antibody confers specificity and allows for washing to remove interfering matrix components, while a detection antibody coupled directly or indirectly to a signal generation system, allows for sensitivity. ELISA assays can detect protein in complex matrices like serum, urine, saliva or plasma often down to the picogram per millilitre range, and numerous commercial kits are available with various degrees of validation. The detection method can involve colorimetric or fluorescence measurement, with fluorescent readouts generally exhibiting increased assay sensitivity. In addition, a horseradish peroxidase activated tyramine signal amplification system, TSA ELAST® (PerkinElmer, Inc) has been shown to increase sensitivity further in traditional ELISA format. The utility of various lanthanide chelates as fluorophores (commercially available in DELFIA® products from PerkinElmer, Inc) offers the ability to run ELISA assays in time-resolved mode, which further reduces potential interference due to the prompt fluorescence of many biological matrix components.

Although considered as a gold-standard in research and analytical laboratories, ELISA suffers from many disadvantages. It can require a large amount of input sample, depending on detection technology used. Moreover, the numerous wash steps make it difficult to automate and labour intensive. These large numbers of manipulations make ELISA prone to variability due to minor difference in procedure and skills between operators.

A method similar to ELISA, but using electrochemiluminescence detection methodology is available from MesoScale Discovery. This technology requires a unique ruthenium-chelate label on the detection antibody which is read via integrated electrodes contained in proprietary microplates. By spotting discrete areas of a well, up to 9-plex analytes can be simultaneously measured, reducing the sample requirement. Application of the Mesoscale platform was recently described in a multicentre study to monitor Alzheimer's biomarkers in cerebrospinal fluid[12].

An alternative to microtiter plate ELISA-type assays is bead-based immunodetection technology using flow cytometry methods. Examples of this approach are the BD™ Cytometric Bead Array from BD Biosciences and Luminex Corp. xMAP® platform, reagents for which are available from various suppliers. The xMAP® system uses 5.6µm polystyrene internally dyed beads coated by specific antibodies to a given analyte. Labelling with two distinct dyes at various ratios allows for the creation of 100 uniquely identified beads that can be individually monitored by standard flow cytometry principles in a dedicated detection instrument. While this allows for potential theoretical multiplexing of up to 100 analytes, rarely more than 15 analytes are validated in single panels due to the

practical complexities of large multiplex assays. Among the various multiplex methods, the Luminex xMAP® platform was generally reported to have better precision than the Cytometric Bead Array[13] or electrochemiluminescence-based Meso-Scale systems[14].

These multiplex technologies require the use of dedicated instruments with limited flexibility for additional analytical applications. Multiplex assays also require complex mixtures of numerous antibodies against a wide variety of biomarkers in the same sample. Although validated panels are generally available for broad applications, these panels might not contain specific targets of interest, especially if an unbiased selection strategy was employed for biomarker identification. In addition, assay performance may not be optimal for each analyte, restricting the lower limit of detection for some targets in the panel. For these reasons, multiplex assays are often used to get a larger picture of biomarker modulation which is then used to select a smaller subset of more relevant targets.

The Alpha Technology platform from PerkinElmer (eg AlphaLISA® and AlphaScreen®) represents a homogenous no-wash ELISA assay alternative that allows samples to be analysed efficiently without compromising sensitivity in complex biological matrices such as serum, plasma, cell culture supernatants or crude cell lysates. This assay platform is less time-intensive than traditional ELISA, allowing for simple 'add-and-read' protocols that enable automated high-throughput applications for rapid biomarker validation. Alpha assays are also truly miniaturisable, a crucial feature when quantitative detection of analytes must be performed on precious biological samples available only in limited supply. AlphaLISA assays are highly robust when using sample volumes as low as 1µl in total assay volumes of 10µl.

Validated AlphaLISA kits are available to study close to 100 major biomarkers in inflammation, cancer, cardiovascular, Alzheimer's or metabolic diseases. AlphaLISA is a bead-based proximity assay, whereby the target is recognised by specific antibodies on two different beads (**Figure 2**).

References

1 Biomarkers: A Market Insight Report (2008). Research Impact, MIR003.
2 Biomarkers Definitions Working Group (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69, 89-95.
3 Eck, SL, Paul, SM (2010). Biomarker qualification via public-private partnerships. Clin Pharmacol Ther 87, 21-23.
4 Wagner, JA et al (2010). The Biomarkers Consortium: practice and pitfalls of open-source precompetitive collaboration. Clin Pharmacol Ther 87,539-542.
5 Marrer, E, Dieterle, F (2008). Biomarkers in oncology drug development: rescuers or troublemakers? Expert Opin Drug Metab Toxicol 4, 1391-1402.
6 Kulasingam, V et al (2010). Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer. Nat Rev Cancer 10, 371-378.
7 Mattes, WB et al (2010). Research at the interface of industry, academia and regulatory science. Nat Biotech 28, 432-433 and numerous related articles in the same issue.
8 Marrer, E, Dieterle, F (2010). Impact of biomarker development on drug safety assessment. Toxicol Appl Pharmacol 243, 167-179.
9 Bhattacharya, S, Mariani, TJ (2009). Array of hope: expression profiling identifies disease biomarkers and mechanism. Biochem Soc Trans 37, 855-862.
10 Hoefkens, J (2010). Towards unbiased biomarker discovery. Drug Discovery World 11, 19-24.
11 Poschmann, G et al (2009). Cell-based proteome analysis: the first stage in the pipeline for biomarker discovery. Biochim Biophys Acta 1794, 1309-1316.

Continued on page 22

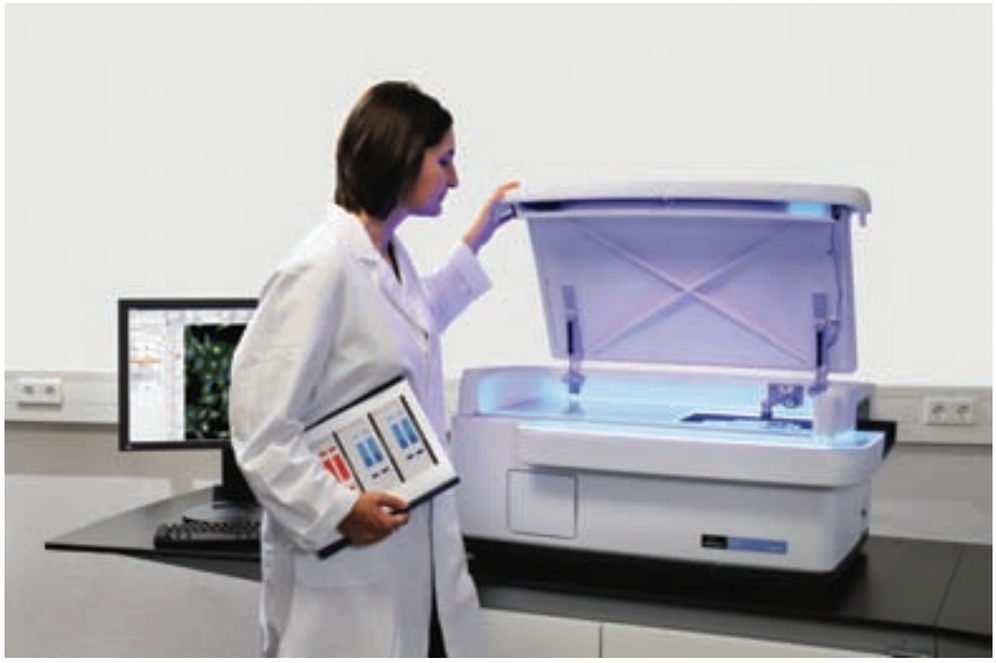

Figure 3: The Operetta® instrument, a high-throughput imaging tool

Analyte binding serves to bridge the beads, bringing them into close proximity resulting in chemical energy transfer and creation of a strongly amplified luminescent signal with high sensitivity and large dynamic range. This signal can be read on versatile multilabel detection instruments such as the EnVision® multilabel reader or a lower cost dedicated EnSpire™ reader, both available from PerkinElmer Inc. Flexibility of Alpha technology for development of custom biomarker assays is achieved through availability of a variety of toolbox beads pre-coated with common affinity tags, or uncoated beads that can be used for direct conjugation of capture antibodies by end-users. A selection of more than 60 validated AlphaScreen SureFire® kits are also available to measure activation of kinases, signalling proteins and transcription factors in cell-based assays.

In conjunction with biochemical assays, cellular imaging analysis is increasingly used in biomarker research. Immunocytochemistry and immunohistochemistry can monitor level and localisation of biomarkers in cells and tissue sections, where the antibody is typically coupled to an enzyme, fluorophore or quantum dot in order to generate a visual readout. The possibility to monitor the protein of interest in specific cells and to gain localisation information by confocal microscopy make cellular imaging a powerful tool for biomarker applications, however data generated by this technique has historically been more subjective than quantitative, and opportunities for automation have been somewhat limited. Recent development of microplate-based high content imaging solutions interfaced to powerful data analysis software in systems such as the Opera® and Operetta® instrument from PerkinElmer Inc (**Figure 3**) enables high throughput imaging biomarker analysis for cellular models. In addition to measuring quantity and localisation of specific targets, critical cellular characteristics such as shape, nuclear morphology, and membrane integrity can also be accurately integrated to provide a comprehensive view of relevant biologies in intact cellular systems.

Label-free measurement: mass spectrometry

Immunoassay methods are restricted by the availability of good quality antibodies that exhibit optimal specificity and avidity for their targets. However, specific epitopes can be modified either *in vivo* or during sample preparation (ie through post-translational modifications or proteolytic cleavage). While a particular antibody pair may identify a subset of the specific target population, multiple antibodies may be required to quantitate global levels of any particular biomarker. This can explain major discrepancies in measured levels of a given target using various kits developed by multiple providers, even when using the same assay

blank

Continued from page 20

12 Lewczuk, P et al (2010). Soluble amyloid precursor proteins in the cerebrospinal fluid as novel potential biomarkers of Alzheimer's disease: a multicenter study. Mol Psychiatry 15,138-145.
13 Richens, JL et al (2010). Quantitative validation and comparison of multiplex cytokine kits. J Biomol Screen 15, 562-568.
14 Chowdhury, F et al (2009). Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. J Immunol Methods 340, 55-64.
15 Lange,V et al (2008). Selected reaction monitoring for quantitative proteomics: a tutorial. Mol Syst Biol 4, 222.
16 Addona, TA et al (2009). Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 27, 633-641.
17 Hüttenhain, R et al (2009). Perspectives of targeted mass spectrometry for protein biomarker verification. Curr Opin Chem Biol 13, 518-525.

technique. As such, appropriate validation, not only of a target or a technology, but of a specific kit is required as the validity of a biomarker in a specific disease state can be highly dependent on the antibody pair involved.

A powerful label-free technique which continues to be effectively applied to biomarker research is mass spectrometry (MS). In its various configurations – quadrupole MS/MS, SELDI-TOF or LC-MS – this technology operates independently of exogenous reagents to impart specificity or sensitive detection of the target. Furthermore, MS allows for the simultaneous measurement of multiple analytes making direct multiplex assays feasible.

Historically, limitations in sensitivity and throughput as well as interference from abundant proteins in complex biological matrices have been significant issues in the adoption of MS for biomarker screening and validation, however advances in instrumentation and analytical protocols continue to make the technique more accessible to these applications. As comprehensively reviewed by Lange[15],selected reaction monitoring (SRM) is a method commonly used for mass spectrometric analysis. SRM delivers a unique fragment ion that can be monitored and quantified in the midst of complicated background matrices, and therefore allows for targeted detection of previously identified proteolytic peptides. Using rigorous analytical protocols, the Clinical Proteomic Technology Assessment for Cancer Network has demonstrated that SRM allows for precise and reproducible measurement of spiked proteins in human plasma[16]. SRM techniques can allow for generic assay development that may be applicable to most protein biomarkers, increasing the feasibility of MS as a time-saving approach over development of new antibody-based assays.

A major issue in adoption of MS techniques for larger-scale biomarker identification and validation efforts relates to sample preparation bottlenecks negatively impacting throughput. Automated systems have been developed to address this issue, such as the RapidFire ® system from Biocius Life Sciences Inc, which utilises small scale solid-phase extraction cartridges containing various chemistries that allow for sample preparation in a wide range of applications. Using this system, increased analytical throughput of up to 20-fold has been achieved.

Although challenges remain with respect to sample preparation and measurement of low-abundance proteins or small molecular-weight analytes in the presence of complex biological matrices such as blood plasma, many improvements continue to be described[17] that make mass spectrometry an exciting technology with increasing utility in biomarker research.

Conclusions

In this article we provide an overview regarding the application of biomarkers to current research and drug development activities. This area encompasses a broad spectrum of end-uses, analytical strategies, and platform technologies – however common requirements for appropriate use of biomarkers include the need for their validation against the biology of interest, as well as the availability of sensitive and simple assays for their measurement. As new biomarkers are identified, technology improvements applied to gene expression profiling, quantitation of protein analytes, and imaging will continue to actively support this field for ongoing development into the future. DDW

Stéphane Parent *is Principal Scientist and* ***Dr Gregory Cosentino*** *is R&D Director within Biodiscovery at PerkinElmer. They have been involved in the development of numerous AlphaLISA kits for the detection of biomarkers in biological samples.*