

## Emerging Label-Free Technologies

### Reducing Failure Due to Toxicity Is Among Challenges Addressed by Recent Offerings

K. John Morrow Jr., Ph.D.

Cell-based assays are powerful tools for monitoring response to external activation signals in living cells. Recognition of this fact has been responsible for roughly 40% growth in the industry in 2008, according to SMI Conferences, which recently sponsored a symposium on the topic. The meeting showcased new developments, including many different automated platforms, new assays for hepatotoxicity, and assays based on the application of transient gene expression, transmembrane receptors, and pluripotent stem cells.

“Of the many reasons for escalating clinical costs, failure due to toxicity holds the promise of being successfully addressed with new, discovery-stage tools,” said Katya Tsaïoun, Ph.D., president of **Apredica** ([www.apredica.com](http://www.apredica.com)). Dr. Tsaïoun argued that while technologies such as toxicogenomics and high-content imaging have the potential to hold down costs in the long run, predictive assays could speed clinical trials and lower their costs in a much shorter time frame.

According to Dr. Tsaïoun, toxicity is a chronic and growing problem in drug development. She cited 12 drug withdrawals from the market between 1999



Routine seeding of human embryonic stem cells in multiwell plates for automated drug screening at Cellartis

## News MOLECULAR DIAGNOSTICS

### Vermillion to Reorganize

Vermillion's ([www.vermillion.com](http://www.vermillion.com)) Chapter 11 plan of reorganization has been accepted by creditors. “The strong vote of support for our plan of reorganization is a major milestone for Vermillion and its shareholders,” says Gail S. Page, executive chairperson of Vermillion's board of directors. “This vote shows we have achieved strong momentum toward emergence from bankruptcy and have positioned Vermillion to establish itself as a leader in high-value diagnostics.”

Vermillion develops tests to diagnose, treat, and improve outcomes for oncology, hematology, cardiology, and women's health concerns. The company's OVA1 test is a qualitative serum test that utilizes five established biomarkers and an algorithm to determine the likelihood of malignancy in women with pelvic mass for whom surgery is planned.

### Response Genetics Inks Distribution Agreement with Genetic Technologies

Genetic Technologies will become the exclusive distributor in Australia, Indonesia, Malaysia, the Philippines, Singapore, and Thailand for Response Genetics' ([www.responsegenetics.com](http://www.responsegenetics.com)) ResponseDX: Colon™, ResponseDX: Lung™, and ResponseDX: Gastric™ genetic-test panels.

ResponseDX tests are PCR-based tests used to analyze the expression of genes that correlate with response to commonly used chemotherapy agents. By personalizing care based on a tumor's genetic makeup, ResponseDX tests can reportedly help physicians to better tailor treatment for their patients with cancer.

ResponseDX genetic tests are available in the U.S. through direct sales and through NeoGenomics Laboratories, the exclusive national clinical-reference laboratory authorized to offer Response Genetics' tests. All tests are performed through Response Genetics' CLIA-certified laboratory.

### Horizon Discovery Sets Up Companion Diagnostic Subsidiary

Horizon Discovery ([www.horizondiscovery.com](http://www.horizondiscovery.com)) launched a subsidiary, Horizon Dx, to support the development of companion diagnostics, initially in the cancer field. The new segment will exploit Horizon's X-MAN™ panel of human isogenic cell models and normal controls.

The X-MAN cell models have been developed to represent defined cancer-patient populations and their matched, normal genetic backgrounds. The technology hinges on the company's Genesis™ gene-engineering platform, which allows the alteration of any endogenous gene loci in human cells. Horizon claims the technology means it can generate in vitro cell models comprising any SNP or activating point mutation.

The first products launched commercially by Horizon Dx will be a source of genetically defined mutant and normal human DNA along with isogenic cell admixtures in formalin-fixed, paraffin-embedded samples. Horizon claims the products will allow companion diagnostic kit developers to validate and standardize assay performance.

“The initial focus of Horizon Dx will be on evaluating new diagnostic kits in partnership with key sector players and supplying

isogenic human mutant versus normal DNA controls to end users via a premier distribution partner,” explains Darrin M. Disley, Ph.D., Horizon's chairman. “The second phase of the business model is to expand the work Horizon is already undertaking with academic and industrial partners to identify novel genomic, proteomic, metabolomic, and other in vitro and in vivo biomarkers and disease signatures from X-MAN models that can form the basis of developing new companion diagnostics in partnership.”

### Predictive Biosciences Acquires CLIA-Certified OncoDiagnostic Lab

Predictive Biosciences ([www.predictivebiosci.com](http://www.predictivebiosci.com)) acquired OncoDiagnostic Laboratory (ODL; [www.oncodiagnostic.com](http://www.oncodiagnostic.com)), a private CLIA-certified anatomic pathology and molecular diagnostics lab. It provides Predictive with a fully integrated pathology laboratory through which the company will commercialize its noninvasive molecular cancer tests.

The first in Predictive's portfolio of assays for cancer management is a urine-based biomarker test for the detection of bladder cancer. The company expects commercialization during this year.

ODL, which was founded in 1985 by a group of pathologists to better serve the growing needs of office-based urologists and other subspecialty physicians, will continue to operate from its Cleveland, OH, headquarters. ODL currently employs approximately 40 professionals, including a national sales force that is supported by company pathologists, laboratory staff, and

and 2001, three of which were due to toxicity that was missed during the development stage. A particularly egregious example was the antibiotic Trovafloxacin, which was withdrawn from the market due to its association with hepatotoxicity.

Studies have found animal testing to be only 50% accurate in predicting hepatotoxicity, with a large number of false positives and false negatives. “Improving hepatotoxic predictivity with an assay of 60% sensitivity and 90% specificity would save at least \$150 million per year,” Dr. Tsaïoun stated.

Hepatotoxicity is a poorly understood phenomenon; in some cases, patients may worsen even after treatment with a suspected agent is terminated. Tests that look for reactive intermediates generated by drug metabolism have proven inadequate for the task since they look at only a single mechanism. Dr. Tsaïoun has developed a multiparameter assay that evaluates a number of markers and is predictive of hepatotoxicity even in the absence of cellular death. This approach encompasses a number of features, including cell loss, nuclear morphology, DNA content, cell membrane permeability, mitochondrial membrane potential changes, and cytochrome C release from the mitochondria.

Dr. Tsaïoun and her colleagues use quantitative algorithms that score responses from 0 to 100%. The methodology was validated using several agents known to be or not to be hepatotoxic, which provided a well-founded set of values on the basis of which each agent can be ranked.

“We believe that in vitro approaches with human cells are powerful tools for evaluation of the hepatotoxicity risk of early

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leads,” Dr. Tsaioun stated. “No one readout or assay is sufficient to definitively predict hepatotoxicity, but a multiparameter cell-based approach can yield insights when single mechanism readouts fail. Certainly, they are more cost-effective at this stage of development than animal studies and potentially more accurate.”

### Transient Transfection

James Brady, Ph.D., director of technical applications at MaxCyte ([www.maxcyte.com](http://www.maxcyte.com)), believes that transient transfection systems offer a host of advantages that have largely been ignored. The company’s MaxCyte STX® system is used for developing cell-based assays. “We prefer transient transfection over stable transfection for a variety of reasons,” he said. “These include decreased time and cost of production, better control of the expression process, and the ability of the system to handle expression of toxic gene products.”

The MaxCyte system uses an electroporation technology designed to move small molecules, antigens, and nucleic acids into all varieties of cells. Using the green fluorescent protein gene as a convenient marker, Dr. Brady demonstrated that a number of well-known cell lines such as VERO, NIH 3T3, and CHO could be transfected with greater than 90% efficiency.

Following transfection, cells can be stored and recovered through cryopreservation with minimal loss of activity. Dr. Brady described a number of case studies in which different targets were successfully transfected such as the  $\beta_2$  adrenergic receptor into CHO cells and the M1 muscarinic receptor into HEK 293 cells. Transient and stably transfected cell lines show comparable levels of performance, he added.

Ion-channel expressing stable cell lines are especially problematic, thus representing an apt target for the MaxCyte technology. Their construction and validation is time consuming and expensive, and because expression of multiple subunits may involve a number of different genes (up to four in some cases), multiple selection with a number of antibiotics could be required. The presence of several antibiotics can lower cell viability and performance.

Using data shared by MaxCyte STX customers, Dr. Brady illustrated how multi-subunit calcium channels can be expressed efficiently in transiently transfected HEK 293 cells. He also showed how the transfected cells can be used to screen ion-channel inhibitors using an automated calcium flux assay that is widely used for high-throughput screening.

### GPCR Ligands

GPCRs are a tempting target for cell-based assays. Clay Scott, Ph.D., associate director for lead generation at AstraZeneca ([www.astrazeneca.com](http://www.astrazeneca.com)), discussed his company’s pursuit of assays that will identify the ligands that bind to this family of transmembrane receptors.

GPCRs play a critical role in the detection of molecular signals from outside the cell that activate signal-transduction pathways and cellular responses.

Dr. Scott and his colleagues compared optical- and impedance-based biosensors for their ability to detect ligand binding to the GPCRs. According to Dr. Scott, impedance-based assays measure changes in elec-

trical impedance (roughly equivalent to resistance) relative to a voltage applied to a cell monolayer.

Optically based methods, on the other hand, quantify the shift in wavelength of reflected light that occurs due to the refractive properties of the biomass. As the morphology and mass redistribution of the cell changes in response to GPCR-ligand driven dynamics,

these events can be detected by the two technologies. However, the AstraZeneca team investigates questions of the suitability these approaches for drug and pharma discovery.

In comparisons of GPCR agonists and antagonists, Dr. Scott noted that, in general, similar values were obtained with optical- or impedance-based assays. Both platforms pro-

See Label-Free Technologies on page 44

## SensoLyte® 520 Calpain Assay Kit Ultra-Sensitive



When it comes to assay kits, ultra-sensitivity is a good thing. AnaSpec is pleased to introduce the new Sensolyte® 520 Calpain assay. This high performance assay uses a novel FRET substrate, its cleavage by Calpain is monitored in the green emission range of 520 nm, making it the industry’s longest wavelength assay.

- Homogeneous assay
- Employs novel FRET substrate 5-FAM/QXL™ 520 (Ex/Em=490/520 nm)
- Measures Calpain 1 & 2 activities
- Linear range of 32.5 to 5000 ng/ml for Calpain 1

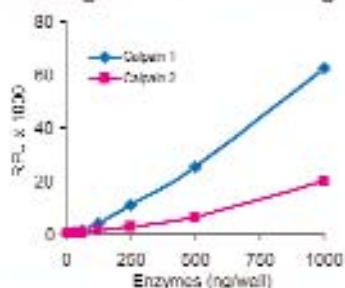


Fig. 1. 5-FAM/QXL™ 520 FRET substrate validated with Calpain isolates. Fluorescence measured 30 min. after substrate incubation with serially diluted Calpain 1 and Calpain 2.

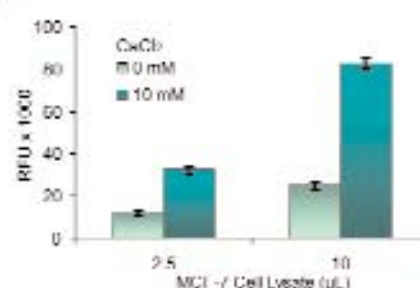


Fig. 2. MCF-7 lysate incubated with 5-FAM/QXL™ 520 FRET substrate for 30 min. Addition of CaCl2 showed increased activity.

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# Label-Free Technologies

Continued from page 43

vide sensitive, label-free precise measurement of these agents' effects on cellular-response. However, using the impedance-based CellKey assay system (MDS Analytical Technologies; www.mdsciex.com), novel data was generated, including temporal responses that distinguish Gi, Gq, and Gs signaling.

Johannes Pschorr, Ph.D., European application scientist for MDS Analytical Technologies, also discussed the CellKey platform. "The system measures impedance changes occurring in response to stimulation or activation of signaling pathways within the cell. A monolayer of cells is seeded into a custom microplate that contains electrodes patterned at the bottom of each well. The CellKey system applies small voltages across a range of frequencies, measuring changes due to transcellular and extracellular current, reported kinetically for each well."

Dr. Pschorr's team is confident that these physiological changes occur as a direct result of signaling-pathway activation. The platform offers the sensitivity to measure functional activity of endogenous targets in live cells, as opposed to being limited to detecting overexpressed recombinant expression systems, he said. Additionally, he reported that the platform has the ability to measure any cell type—adherent and nonadherent cell lines and primary cells.

Analyzing receptors that are expressed with the appropriate accessory proteins gives researchers a picture of receptor function in a physiologically relevant environment. This flexibility allows researchers to conveniently screen a wide variety of targets with a single instrument platform, which helps standardize data to support lead compound selection.

One of the significant challenges Dr. Pschorr faces is the complexity of GPCR pathways and their interdependencies. "When screening for receptor modulators, we need to use methods that are as close to the actual cellular response as possible. Trying to interpret results from cells that are not physiologically relevant can lead to less-than-optimal lines of investigation to pursue."

According to Dr. Pschorr, combining the ability to measure a whole live-cell assay with the ability to measure multiple signal pathways within the cell provides a more informative data result. He predicted that the measurement of integrated response of whole cells rather than measurement of one specific point in one specific pathway will lead to a more thorough understanding of complex receptor activity and compound mechanisms of action.

"The ability to do so in a label-free manner also provides an advantage, both in enabling the universality of the measurement and in allowing researchers to have a robust system to easily weed out nonspecific effects due to labels of the currently employed technologies."

## Human Embryonic Stem Cells

"Human stem cells can be used as a tool in small molecule screening," said Paul Andrews, Ph.D., senior scientist in the drug discovery unit at the University of Dundee,

"but care must be exercised."

Dr. Andrews is the head of a program that exploits stem cells to serve as physiological-relevant cell models of disease. As Dr. Andrews explained, embryonic stem cells have an unlimited capacity for differentiation, whereas adult stem cells are more circumscribed in this respect. A third option is induced pluripotent stem cells, which are derived from reprogrammed adult somatic cells, and are similar to embryonic stem cells. Being activated by small molecule chemical signals, they offer an opportunity to understand and manipulate developmental events. This is only sometimes true (e.g., in the case of retinoic acid) but the majority of other cases involve protein ligands (e.g., the BMPs, Wnts, and Activins).

Small molecules are useful because they may act as surrogates for the protein ligand by stimulating the pathways or inhibiting a negative regulator. In some cases a series of steps may require a toolbox of molecules to bring about the final differentiated state.

A host of challenges are posed by human stem cells if they are to become an integral part of drug discovery. These include the requirements of feeder-free technology and a need to maintain long-term stability of cultures. At present, the understanding of cellular commitment, the steps required for appropriate differentiation, and avoidance of cell death and heterogeneity are all significant hurdles, according to Dr. Andrews. Nonetheless, stem cells constitute a powerful model for the study of disease states and predictive toxicology.

The stem cell technology program includes several other institutions under a £9.5 million multicenter umbrella pursuing cell delivery, high-content analysis, building of libraries,

and development of gene-reporter systems. Cellartis (www.cellartis.com) supplies more than one billion cells per week, generated through large-scale automated production. The cells are plated in either 96- or 384-well plates and tested against a large collection of compounds. Included are small, drug-like molecules targeting ATP sites on protein kinases, marketed drugs, bioactives, and epigenetic modifiers.

After processing, the cells are imaged by immunofluorescence and analyzed to quantify a particular marker (e.g., fluorescence due to the activation of reporter genes or the levels of a protein detected by antibody staining).

Positive hits in the initial screening are followed up through potency testing, kinase profiling, mechanism of action studies, toxicity, and gene-expression profiles. One of the targets Dr. Andrews and his team have focused on is the bone morphogenetic proteins, or BMPs, which are members of the TGF $\beta$  superfamily of extracellular signaling molecules. They observed strong effects on Oct3/4, one of the master regulatory transcription factor proteins that are essential to maintain pluripotency. Focusing on the ability of the BMP4 to drive differentiation, they identified a number of small molecular antagonists that block this effect and maintain pluripotency.

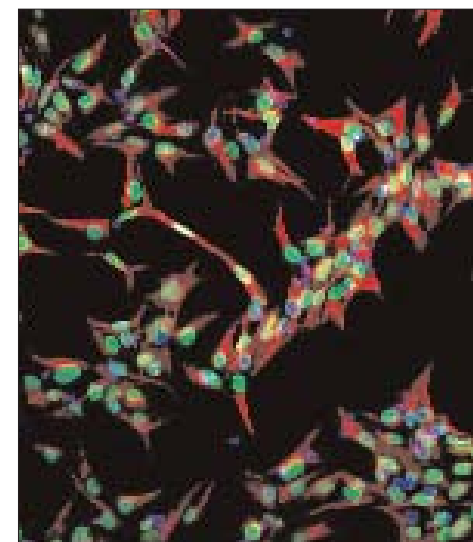
"We sought to identify both the Oct3/4 upregulating and downregulating agents as early differentiation-promoting factors," Dr. Andrews said. By screening their kinase libraries and bioactive libraries they identified a number of candidates. Three compounds are currently undergoing follow-up work to determine their mode of action and have shown good efficacy in replacing the endogenous

BMP inhibitor Noggin in neuro-ectoderm induction protocols.

Other current and future activities include identification of early differentiation factors, and an induced pluripotent stem cell drug discovery program. "In the future we will need effective mode-of-action studies to reveal new pathways and targets," Dr. Andrews concluded.

The approaches profiled in this article are among the most promising possibilities for accelerating the drug discovery process and saving time and financial resources. There is a pressing need for new technologies as drug development has lagged, in many cases due to the failure to recognize fatally flawed compounds early in the discovery process. New cell-based assay strategies may hold the key to breaking one component of the discovery roadblock.

GEN



Human embryonic stem cells growing in feeder-free culture conditions: Microtubules are shown in red, Oct 3/4 pluripotency transcription factor in green, and DNA in blue. University of Dundee

## Assessing Gene Function in Real Time

One of the challenges in RNAi research is choosing an appropriate robust cell-based functional assay for obtaining important information about the phenotypic effect of targeted gene knockdown, discriminating off-target and toxic effects. Both off-target interactions and toxicity are an inherent part of the RNAi approach, dependent on the RNAi target sequence, concentration, and cell type used.

In a new application note ("Using the xCELLigence™ System for Functional Genomics: Assessing Gene Function in Real Time"), investigators from Roche Applied Science (www.roche-applied-science.com) describe use of the instrument, which allows for label-free continuous monitoring of changes in cell phenotype using microelectrodes to record electrical impedance, for assessing RNAi-mediated knockdown of gene function.

Cells were seeded in standard 96-well microplates, manufactured with integrated microelectronic sensor arrays called E-Plates 96. The interaction of cells with the electronic biosensors generates cell-to-electrode impedance responses, measuring many aspects of cell status, including cell number, cell viability, cell morphology, and the quality of cell attachment. Real-time, continuous measurement results in uninterrupted documentation of cell phenotype

by producing time-dependent cell response profiles (TCRPs), according to the Roche team, who add that TCRPs provide predictive information about how cells and cellular pathways are responding in vitro.

Kinetic information revealed by TCRPs can discriminate siRNA-mediated off-target and toxic effects. The focus in this application note was on several genes implicated in the mitotic pathway.

HeLa and A549 cells, obtained from ATCC, were maintained in DMEM media with 10% FBS and 1% penicillin and streptomycin, at +37°C with 5% CO<sub>2</sub>. Cell attachment, spreading, and proliferation were continuously monitored every 30 minutes on E-Plates 96 using the xCELLigence system.

The electronic readout of cell-sensor impedance is displayed continuously in real-time as the cell index (CI). The CI value at each time point is defined as Rn-Rb/Rb, where Rn is the cell-electrode impedance of the well with the cells, and Rb is the background impedance of the well with media alone.

### Assessing Gene Function after Knockdown

To better document the correlation between target gene expression, CI changes, and mitotic arrest, threefold serial diluted KIF11 siRNA (KIF11-7904) was transfected into HeLa cells in E-Plates 96 for cell index measurements, and 16-

well chamber slides for quantifying mitotic index; 24 hours post transfection, cells were harvested from E-Plates 96 to quantify KIF11 mRNA levels, or fixed and stained using p-H3 antibody in chamber slides to determine mitotic indices. TCRPs showed time- and concentration-dependent dose response curves, with higher siRNA concentrations producing more CI changes.

The kinetics of CI changes were very similar for the different concentrations of siRNAs. CI values for transfected samples diverged from control samples, starting 9–12 hours post transfection, reaching the lowest level at 24 hours post transfection, before the CI started recovering. CI changes were highly correlated to target mRNA levels and mitotic indices, with higher siRNA concentrations producing more pronounced CI changes, resulting in higher target downregulation and a higher mitotic index.

Interestingly, the Roche scientists note that siRNA transfected at a concentration of 0.01 nM still produced robust CI changes and target downregulation. Similar dose responses were also observed for PLK1-448 and PLK-450 siRNAs, while no dose response was seen for control siRNAs.

The dose-dependent TCRPs obtained allow for the determination of the efficacy of target knockdown on a particular phenotype, explain the Roche researchers.