HIGH THROUGHPUT SCREENING

meeting the challenges of more cell-based assays and HCS

The use of high content screening and cell-based assays within HTS laboratories is on the increase but there are still many challenges to overcome.

By Sandra Fox, MBA

Each year, HTS laboratories perform slightly more cell-based assays on average than they did the year before. Cell-based assays represent about half of the screening conducted in HTS laboratories worldwide.

Some HTS laboratories are also incorporating high content screening (HCS) within their HTS operations. Although HCS is rarely used in HTS as a primary screen, almost one-fourth of the 54 HTS laboratories surveyed for the latest HighTech Business Decisions Report (*High Throughput Screening 2005: New Users, More Cell-Based Assays, and a Host of New Tools*), say that some HCS was conducted in their laboratory as a primary screen in 2005. As a percentage of all screens, HCS is still relatively small, representing about 4% of all HTS. This is expected to double in the next two years, as shown in **Figure 1**. The use of HCS is increasing primarily because multiple parameters can be measured, the results are more predictive and biologically relevant, a better understanding of the basic biology can be obtained including subcellular events, and more information is obtained to help decide which compounds should be moved forward.

Increasing use of HCS and cell-based assays within HTS laboratories presents several challenges, however.

Challenges: performing HCS in HTS

Performing HCS in a HTS laboratory can be difficult at best. New systems must be purchased and a new expertise must be developed. The data analysis can be overwhelming given the magnitude of the data collected. Imaging systems and analyses tend to make HCS low throughput, and assay development is much more complex as compared to a typical HTS assay. The primary challenges HTS directors face when performing HCS are shown in **Table 1**, in order by number of times these challenges were mentioned in the survey.

A few selected comments regarding the challenges of performing HCS, as provided by HTS directors surveyed at leading HTS laboratories, follow:

"The barriers to HCS are the **need for a new instrument, low throughput, and the learning** curve." *Pharma/Biotech HTS Lab*

"**Information overload** is the barrier. There are still big **problems with data generation, storage, and search-ability** for live cell screens in HCS." *Pharma/Biotech HTS Lab*

"The barriers are **low throughput, cost, data analysis and ability to automate** around HCS platforms." *Pharma/Biotech HTS Lab*

Figure 1

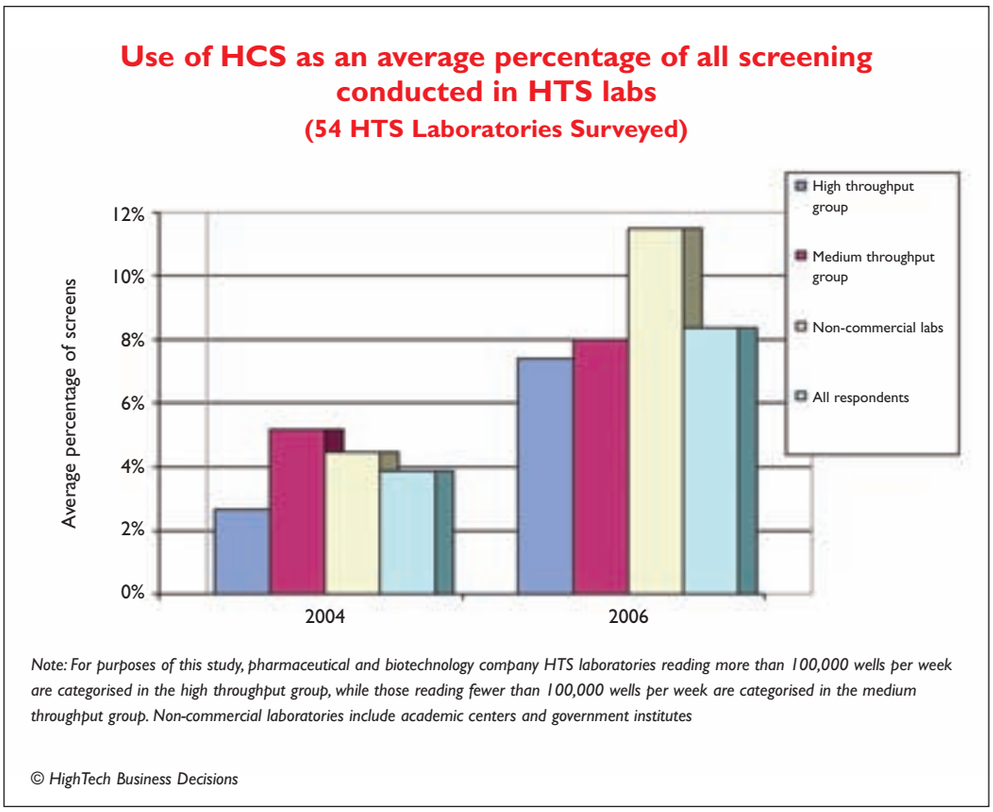

Table 1: Barriers and bottlenecks regarding the use of HCS in HTS laboratories

CHALLENGE
Cost: systems and operators/capital expenditures, resources
Data analysis, data handling, and information overload
Low throughput
Learning curve on new instruments, decisions, validation
Assay development/other ways to screen receptors
Complexity
Incompatibility with 1536-well microplates
Time-consuming to fix cells
Automating around HCS platforms difficult
Data storage and search-ability for live cell screens difficult

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"The barriers to doing HCS include the **unavailability of readers that are high throughput**. Washing and staining are not convenient and they slow down the throughput. It is also an **expensive** technology." *Pharma/Biotech HTS Lab*

"The barriers to HCS are **assay development, data analysis, and data storage**. We need better algorithms for data analysis of our screens. These assays are not really high throughput." *Pharma/Biotech HTS Lab*

"HCS is a big bottleneck. **Understanding the biology is the problem**. When you get the readouts, you have up to **200 data points per well. Which are the most relevant? Which ones do you use to normalise? How do you remove false positives?** It is not easy. This is a great technology and it will be helpful in the future. We should not use HCS alone, though. You need more than one reference point." *Pharma/Biotech HTS Lab*

Challenges: cell-based assays in HTS

The use of cell-based assays also presents major challenges not encountered in simpler biochemical assays. Developing cell lines that reliably express

proteins of interest is a major challenge, with many laboratories experiencing falling expression levels with increasing passages. The primary challenges HTS directors face when establishing high quality cell lines for their cell-based assays are shown in **Table 2**, in order by number of times these challenges were mentioned in the survey.

A few comments regarding the challenges of establishing cell lines for cell-based assays, as provided by HTS directors surveyed at leading HTS laboratories, follow:

"**Getting a line to stably express the protein** of interest is a challenge. The expression of GPCRs has been worked out, but ion channels are a challenge. We have transient expression systems. We freeze large numbers of cells so they are consistent for the life of the HTS run. One advantage is that we get a cell count and we can monitor expression in individual cells." *Pharma/Biotech HTS Lab*

"The major challenge in developing cell lines is **specificity for the target. Secondary challenges are developing lines that will give reproducible results over a range of growth conditions**. For our operation, the signal has to be stable over 16 to 20 hours, so cells from a single lot need to behave well over that time. We typically include reference signals that check for cell viability. If we have compounds that inhibit signal, it may be a toxic compound, so we check that." *Pharma/Biotech HTS Lab*

"Robust signal-to-noise ratios are the largest challenges, **especially in 1536 formats where there are few cells per well**. Some assays and cell-lines scale well; some don't. We mostly look at the actual response in the assay." *Pharma/Biotech HTS Lab*

"Signal-to-noise in the assay itself is the main challenge. I can't believe this hasn't changed in the last five years – I wouldn't have believed this would still be a bottleneck. **It takes a lot of brute force work**." *Pharma/Biotech HTS Lab*

"**Good transient transfection methods would be helpful** and would eliminate the need for stably transfected cell lines and the problems with loss of expression." *Pharma/Biotech HTS Lab*

"A major issue for us is dealing with so many cell lines and having to work out the conditions in **pilot mode, only to have them behave differently in screening mode**." *Pharma/Biotech HTS Lab*

Table 2: Cell-based assay challenges: cell lines

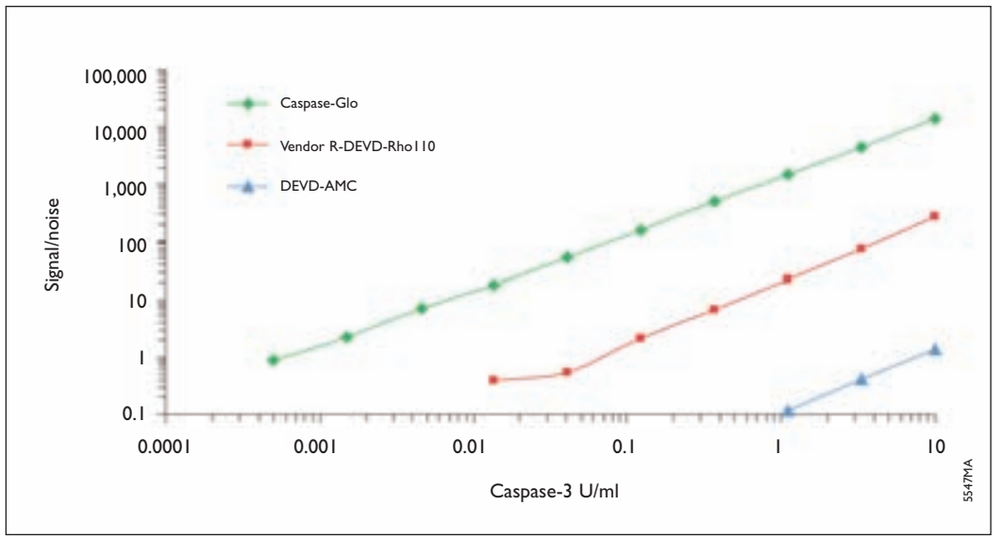
CHALLENGE
Maintaining protein expression levels and stability: (Reducing variability, determining optimal growth conditions, providing sufficient quantities of cells, obtaining reproducible results over a range of growth conditions)
Time and expertise requirements: (Slow expression and growth, finding the right people with expertise, developing the art of growing cell lines)
Obtaining robust signal-to-noise ratios
Target specificity
Using transient versus stable transfections
Phenotypic/clonal drift
Assay development/pilot-to-screen transfer
Relating cell-based assays to biology
Contamination

Suppliers are meeting some of these challenges with a host of new tools as described below.

Easy-to-use database management and analysis tools for HCS

Scientists in HTS laboratories indicate that managing data and making decisions with that data are some of the most difficult challenges of HCS. **Cellomics, Inc**, a business unit within the Fisher Biosciences Group, (www.cellomics.com) has recently developed several products to meet this challenge, including the Cellomics Store database. This is an enterprise class relational database designed to manage, track and archive large volumes of data and images from HCS. Judy P. Masucci, PhD, Director of Marketing and Sales Support at Cellomics, says: "This database makes it easy to find, analyse, visualise and manage all of the HCS data. Our vHCS™ Discovery Toolbox provides powerful PC-based tools to create, manage, analyse, visualise and communicate HCS image, data, information, and results."

The Cellomics BioApplications product, which provides sophisticated image analysis algorithms, allows scientists to make complex measurements and perform analysis of their images in an easy-to-use format. Masucci describes the benefits: "Anyone can make sense of their data using these tools, even if they aren't an imaging expert. And all of these products work together in a seamless, integrated, fashion."



Promega's Caspase-Glo® Assays are up to 1,000-fold more sensitive than fluorescent methods

Cellomics' latest release is a new BioApplication product called Tube Formation, which allows scientists to look at angiogenesis and tube formation in their discovery pathways. The Tube Formation BioApplication provides a direct measure of angiogenesis in a validated functional HCS assay by measuring the morphology of endothelial cells and tubes.

A homogeneous approach to profiling signalling pathways without imaging

Although the use of HCS provides the opportunity to interrogate biological processes at a level of detail not previously possible in industrial screening, it also introduces limitations in throughput, data analysis, cost-effectiveness of screens and requires equipment and personnel unique to HCS. Dr Keith Olson, Director of US Sales and Marketing at **DiscoveRx, Inc** (www.discoverx.com), says that some HCS assays can be achieved with simpler approaches that are easily adapted to HTS programs: "The DiscoveRx PathHunter™ technology provides a homogeneous, non-antibody approach for detection of protein translocations within living cells. This technology enables users to directly monitor key cell signalling events using a chemiluminescence readout that requires no imaging. It can be automated and miniaturised too for use in HTS labs."

DiscoveRx has launched the PathHunter cell lines and detection reagents for profiling signalling pathways without sophisticated imaging equipment. Translocation is measured directly, without lengthy incubation times associated with reporter gene approaches, and non-specific affects are minimised. Olson explains: "PathHunter is based on positional complementation of the beta-galactosidase enzyme within specific sub-cellular compartments in whole cells. PathHunter can generically be applied to any target protein that is thought to show nuclear translocation, including proteins that are not directly involved in active transcription, such as proteases, phosphatases and kinases. Future generations of the PathHunter product line will be capable of monitoring other types of intracellular translocation events as well, such as cytoplasm-to-membrane translocation, and even extracellular detection of secreted proteins."

The technology can be delivered either as ready-to-use stable cells expressing a target protein of interest, or as an open system allowing users to investigate their own proprietary drug targets. Olson describes some of the benefits: "The PathHunter system requires no fluorescence imaging or antibodies and the chemiluminescence detection limits library interferences. In addition, it has a high signal-to-background ratio and robust performance with a Z'-factor > 0.70. Since the signal is generated using enzyme amplification, PathHunter also permits users to study exogenously expressed proteins at cellular expression levels at or below the endogenous protein, which minimally perturbs the natural balance of cellular signalling cascades."

A label-free system for measuring endogenous receptors in cell-based assays

MDS Sciex (http://www.mdssciex.com/products/aboutcds/) recently launched the label-free, cell-based CellKey™ System to meet two main

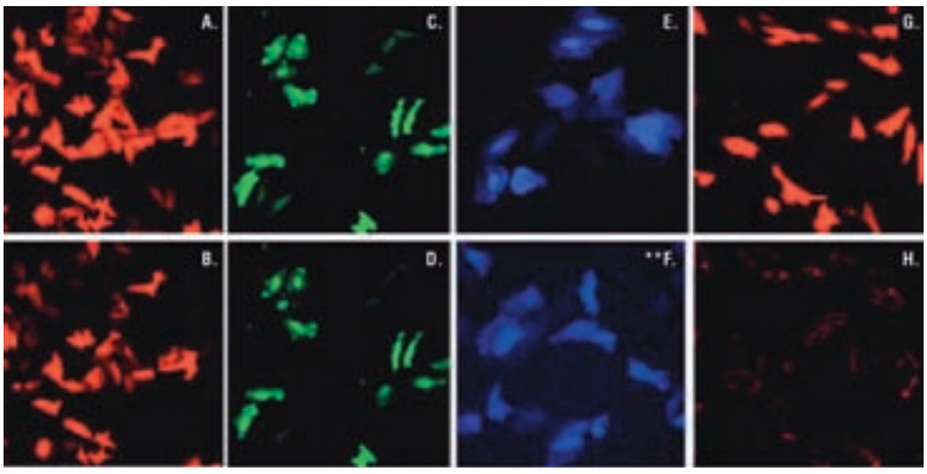
challenges encountered by drug discovery scientists performing cell-based assays for targets such as cell surface receptors. Anne T. Ferguson, PhD MBA, Product Manager at MDS Sciex, explains the problems these scientists face: "One problem is creating stable cell lines overexpressing the target of interest. This process is time consuming and sometimes unsuccessful because overexpression of the target may be toxic to the host cell. If a cell line can be generated, it may not stably express the target gene of interest, thus producing variable results over time. Lastly, there are intellectual property issues for some popular targets, which prevent development of a model system to use for screening purposes."

A second concern for scientists in drug discovery is that data from current cell model systems are not biologically relevant because target function is not measured in the endogenous setting and in a disease relevant cell context. For many current cell-based assays, overexpression of the target gene is required to achieve a robust and reproducible response. This genetic manipulation can cause aberrant or artifacts in signal transduction and may lead to screening data that is not representative of the natural function of the receptor. In addition, receptor activity is typically analysed in a cell type that can be efficiently transfected or easily infected such as HEK 293 or CHO cells, cell types not appropriate to use as models for most diseases.

Ferguson explains how the new CellKey™ System addresses these challenges: "Because the system is based on a detection method called impedance, which is a direct measurement of cellular activity, it circumvents the need for the use of fluorescence, luminescence or absorbance-based probes. The CellKey™ assay is a real time, kinetic, live cell assay for measuring cell surface receptor activity and performing hit confirmation, pharmacology and receptor selectivity analysis. Additionally, it provides information on the pathway through which the receptor signals by generating impedance-based responses that are unique to Gs-, Gi- and Gq-protein coupled and tyrosine kinase receptors.

"Whether in cell lines or primary cells, the system consistently and robustly measures endogenous receptors, which obviates the need to transiently or stably transfect cells and

Promega's HaloTag™ ligands withstand fixation



HeLa cells were transiently transfected with either the HaloTag™ expression vector or a red IFP (DsRed; BD BioScience Clontech Cat. # 6974-1). Twenty-four hours later, cells expressing the HaloTag™ protein were labelled with HaloTag™ TMR ligand (5µM, **Panels A** and **B**), HaloTag™ diAcFAM ligand (10µM, **Panels C** and **D**), or the blue ligand (currently under development; 25µM, **Panels E** and **F**) for 15 minutes at 37°C/5% CO_2. Unbound ligand was washed from cells, and cells were incubated for 30 minutes. Live cells were imaged and then cells were fixed with warm 4% paraformaldehyde plus 0.4M sucrose in PBS for 10 minutes. Fixed cells were imaged, again using identical settings for fluorophore excitation and detection of emission light. **Panel G** shows staining of live cells expressing the multimeric DsRed IFP. **Panel H** shows the same cells after fixation. **Panels A, C, E** and **G** represent cells imaged before fixation; **Panels B, D, F** and **H** were imaged after fixation. ** Note: Images in **Panels E** and **F** were captured with a CCD camera and represent different fields of view within the same culture well. All other images were captured with a confocal microscope and show identical fields of view before and after fixation

enables secondary screening in a cell type that represents the disease in which the target is relevant," Ferguson says.

CellKey™ is a trademark of MDS Inc through its MDS Sciex Division. The full product suite includes an instrument, special microplates for the impedance-based detection, and software that contains a proprietary data analysis package. Currently, the system has been used to measure more than 100 different types of endogenous receptors in the context of more than 25 different cell types including primary cells.

Rapid labelling in live- or fixed-cell populations

New options for rapid, site-specific labelling of proteins in living cells and *in vitro* are provided by **Promega Corporation**'s (www.promega.com/drugdiscovery) HaloTag™ Interchangeable labelling Technology. With a single genetic construct, this technology enables cellular imaging and *in vitro* protein analysis. Neal Cosby, PhD, Manager for Drug Screening at Promega, says: "The technology is based on the formation of a covalent bond between an expressed HaloTag fusion protein of interest and synthetic ligands that carry a variety of functionalities, including fluorescent labels, affinity tags and solid phase attachments. The covalent bond is essentially irreversible, yielding a complex that is stable even under denaturing conditions."

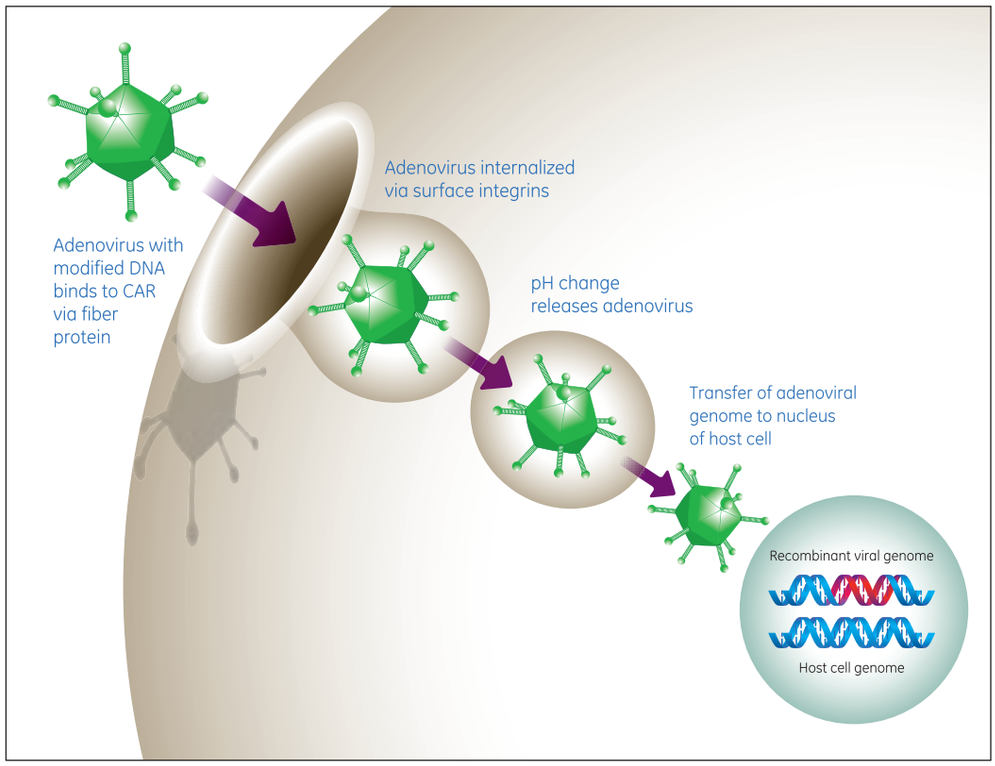
Current localisation methods with fluorescent protein can be limiting for high content analysis. Cosby explains the benefits of HaloTag: "The ability to create covalently labelled HaloTag fusion proteins allows researchers to image and localise labelled HaloTag protein fusions in live- or fixed-cell populations. Because the ligand dictates the colour, a single genetic construct can allow protein labelling in a variety of colours."

Luciferase-based live cell substrates

Promega's pGL4 Reporter Vectors are codon optimised for increased expression and decreased background, which enables screeners to obtain data that more accurately reflect the biological systems under study. Cosby continues: "Our EnduRen™ and ViveRen™ Live Cell Substrates, for use with Renilla Luciferase, are the first luciferase-based live cell substrates for kinetic reporter gene analysis." The company's luciferase-based reporter gene technologies (ie, bioluminescence) are routinely used for GPCR and nuclear receptor assays.

Luminescence-based protease assays can relieve screeners and researchers from the restrictions inherent with fluorescent assays. Cosby says: "Our

GE Healthcare



luminescence-based protease assays are easy and highly sensitive enzymatic and cell-based assays. These assays allow for simplified high-throughput screening for protease inhibition, profiling or apoptosis determination. Fluorescence-based protease assays are subject to inherent background noise that greatly compromises sensitivity. For example, fluorescent compounds present in media or even library compounds themselves can emit light under excitation conditions. Also, any overlap of a fluorophor's excitation and emission wavelengths will reduce assay sensitivity."

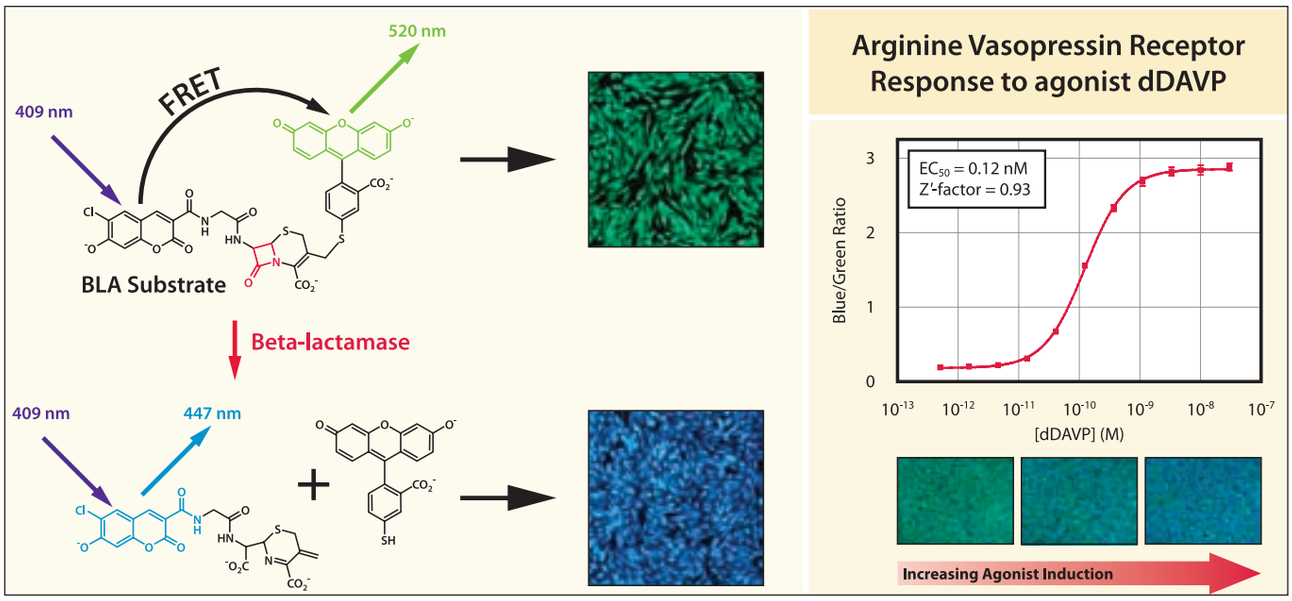
A simple vector gene delivery system in diverse cell types for HCS

GE Healthcare (www.amershambiosciences.com) has developed a range of adenoviral encoded cellular sensors based on protein translocation and reporter gene activation called Ad-A-Gene Vectors. These vectors allow GFP-protein translocation and Nitroreductase (NTR) gene reporter assays to be easily established in a wide range of cell types. Nick Thomas, Principal Scientist at GE Healthcare, explains the benefits: "Ad-A-Gene Vectors overcome the limitations of using stably engineered cells and to enable a more diverse set of assays to be applied in drug screening and lead compound profiling. These convenient, easy to use reagents greatly expand the possibilities of HCS in drug target validation, screening and compound profiling."

Many cell-based assays used for drug discovery use stable cell lines to express genetically encoded sensors such as GFP fusion proteins. While these engineered cells fulfill some of the requirements of HCS, limitations of transfection methods generally restrict sensor expressing cell lines to standard cell types such as HEK293, CHO and U2OS, which may not fully reproduce the biological context of a drug target or signalling pathway. The Ad-A-Gene Vectors provide an efficient and technically simple system for expression of cellular sensors in diverse cell types, including primary cells.

Bob Kendall, Senior Scientist at GE Healthcare, says: "Adenoviral vector mediated transient expression of cellular sensors, either as fluorescent protein fusions or live cell gene reporters, allows rapid assay development to engineer a range of assays. These assays can be used for profiling the activities of candidate drugs across multiple cellular systems selected for biological and physiological relevance to the drug target and disease state."

Ad-A-Gene Vectors can be used with a number of different instruments. Ad-A-Gene NTR reporter gene vectors can be used with plate-



Invitrogen's GeneBLAzer® beta-lactamase technology is a proven method used to study the pharmacological effects of compounds on specific cell-based targets implicated in disease

readers, macro-imagers, and a range of HCS instruments. Ad-A-Gene GFP translocation vectors are well suited for subcellular analysis on HCS platforms.

Ray Ismail, Senior Scientist at GE Healthcare, summarises: "Delivering cellular sensors using adenovirus is quick and easy and does not require any specialised equipment. The Ad-A-Gene Vector is simply added to cells in culture medium and the majority of fluorescent protein translocations and reporter gene expression can be detected within 24 hours after transduction of target cells. This approach allows rapid assay development in the correct physiological context, without the necessity for engineering and selection of stable cell lines."

Because multiple assays can be readily implemented using the range of Ad-A-Gene Vectors, potential compound 'off target' effects can be assessed using sensors covering several different signal transduction pathways with the array of different fluorescent protein tagged gene targets and reporter gene adenoviral vectors offered.

Ready-to-screen cell lines

Few fully validated cell lines for analysing a broad range of disease-relevant signal transduction pathway readouts for automated screening have been commercially available for HTS laboratories. Scientists developing their own cell lines are faced with lengthy assay development times, where several months of development may be required. However, the major challenge has been achieving adequate performance from the cell line, such as a good Z'-factor and correct pharmacology, to support HTS.

Invitrogen Corp (www.invitrogen.com) has developed the ready-to-screen GeneBLAzer® and CellSensor™ Cell Lines for HTS. Jared Browning, Product Manager at Invitrogen, describes the benefits: "These cell lines were developed to cover a broad range of target and disease relevant intracellular signalling pathways. They generate a ratiometric readout for detection that minimises false hits, and they are fully validated to meet the demands of HTS. The FRET-based, ratiometric output minimises experimental noise that can otherwise mask the underlying biological response of interest. The benefits include a reduction of both false positives and negatives, and the ability to distinguish subtle biological responses to stimuli. All of these performance features eliminate most, if not all, of the lengthy and challenging assay development bottlenecks."

GeneBLAzer® and CellSensor™ Cell Lines are tested to demonstrate key performance criteria, including Z'-factor ≥0.6 and correct pharmacology to known stimuli, which is determined by EC_{50}/IC_{50} in comparison to published values. Additionally, Invitrogen tests for DMSO tolerance, cell number, and substrate loading time, and certifies each to be Mycoplasma negative. Browning says: "Researchers can obtain ready-to-use, high performance cell lines without undertaking the risk, time and difficulty of developing them in-house."

Invitrogen provides cell lines for interrogating a

wide array of pathways and targets, including GPCRs (Gs, Gq and Gi-Protein Coupled), kinases (JAK/STAT, MAPK, others), and nuclear hormone receptors (including a panel of steroid hormone receptors such as ERalpha, ERbeta, PPARγ), and other transcriptional regulators. In addition, Invitrogen will develop cell lines tailored to specific requirements through its Custom Assay Services programme.

Invitrogen also provides the Fluo-4NW Calcium Assay Kit, which does not use a quencher dye. Researchers performing automated intracellular calcium flux measurements using other kits and methods often encounter non-specific assay interference due to the inclusion of a quencher dye. This interference may adversely impact the ability to distinguish subtle biological responses to stimuli and can also have undesirable pharmacological effects within the desired assay. Browning describes the benefits: "The Fluo-4NW Calcium Assay Kit provides excellent performance without a quencher dye, combined with the convenience of a no-wash format. This new approach to calcium detection minimises the potential of non-specific assay interference and reduces percent CVs, thereby improving data quality using either adherent or suspension cell formats."

Outsourcing high capacity cell culture

HTS researchers are increasingly out-sourcing several cell-based assay activities to take advantage of cost efficiencies and the expertise of companies in various R&D areas. Dr Charles Saller, CEO of **ABS, Inc** (www.absbio.com), says: "Our expertise in high capacity cell culture for large screening campaigns is an advantage for our clients. We provide cells for scale-up as well as specific cell types such as primary human cell cultures. In the past, these benefits have been limited to cell membrane preparations and various cellular extracts for HTS assays because of the problems associated with shipping viable cells that are ready for use. Over the past year, we have begun initiatives to extend these benefits to live-cell assays, so that clients can save the labour and expense of in-house cell culture. They are then free to concentrate on the assays and not the biological reagent preparation."

For a number of years, ABS has provided a number of clients in New Jersey and eastern Pennsylvania with live-cell deliveries, either in flasks or microtiter plates that are ready for use. These deliveries have now become routine and reliable. Saller describes the expanded service: "We are now offering to set up satellite facilities that are located near our clients' screening facilities so that deliveries can be quickly made on a daily basis. This provides a very cost-effective alternative to costly labour-intensive in-house cell culture production or large capital expenditures for automation equipment. Companies in close proximity to one another, can share the resources of an ABS satellite facility, further reducing costs."

ABS is now in the testing phases of a technology that will extend the benefits of its live-cell services to a much wider range of cell culture HTS researchers. With this technology, cells are frozen in microtiter plates and are ready for use without any media changes or manipulations. Saller explains: "Thus far, cell viability has been excellent with this technology for a variety of cell lines. We are now evaluating the functional capabilities of cells after thawing and plan to launch this service in the near future." DDW

***Sandra Fox** is President and Founder of HighTech Business Decisions, a market research and consulting firm specialising in industry reports for the pharmaceutical and biotechnology industries. The company recently published the report* High Throughput Screening 2005: New Users, More Cell-Based Assays, and a Host of New Tools. *www.hightechdecisions.com*