# Multiplexing for biological relevance in high throughput screening

The multiplexed assay is a technique used increasingly in high throughput screening (HTS) labs to study the complex environment of a target, according to HighTech Business Decisions' (HTBD) industry report *High Throughput Screening: New Strategies, Success Rates, and Use of Enabling Technologies*.

**By William P. Downey, Dr Jennifer Hartigan and Cindy Liu**

Fifty-five directors and managers at pharmaceutical, biotech and non-commercial HTS labs were extensively interviewed about their drug discovery efforts in the industry study. About half of the directors report using a multiplexed format in their high throughput screening. This represents a 55% increase in a two-year period. The study finds that the use of multiplexed assays as a percentage of high throughput screening has also increased, see Figure 1.

The interest in multiplexing is due to its potential to provide a better understanding of a pathway as a whole. A comment from one of the HTS lab directors in the study summarised the situation well: "We would like to use a mix of purified enzymes so we control the entire reaction. It would be great if we could make a sensitive measurement of our targets in extracts of patient samples. If we could look at which compounds do what and take several measurements per sample, then we might get a more biologically relevant readout."

HTS labs indicate they would like to use more multiplexing in the future. They are interested in screening the usual targets, kinases, GPCRs or ion channels, but using a multiplexed approach, for example, assaying multiple kinases to explore compound targeting and selectivity. Assays of multi-step metabolic pathways or cancer-specific multiple events are invaluable for exploring targets in a complex context. The directors also mentioned the importance of using multiplexing to eliminate off-target effects like the species cross-reactivity of a target or cytotoxicity.

Even though multiplexing provides an enriched biological context for the target, these assays can be problematic because of their complexity. Some of the challenges in multiplexing include difficult implementation and optimisation of the assays. More biochemical tools and better signal-to-noise for more robust readouts are among the most mentioned improvements needed for multiplexing. A summarised list of multiplexing improvement needs is shown in **Table 1**.

There is a clear interest in multiplexed assays among HTS directors, particularly as enabling technology becomes available to run complex assays. To support this important discovery endeavour and address some of the challenges of working with complex assays, several companies have developed new multiplexing technologies. These technologies include reagents and reporter genes for differentiated detection, optimised assay conditions to speed the uptake of new technology, and sensitive instrumentation for automated screening accompanied by data analysis tools for managing complex readouts.

## Robust high content screening and optimised assay solutions

Thermo Scientific (www.thermofisher.com) has a fully integrated high content screening and cellular imaging solution that includes fluorescent

reagents, engineered cells, automated imaging technologies and image analysis software. Thermo Scientific's Cellomics® HCS Reagent Kits provide easy-to-use validated kits, with methods and reagents to prepare imaging-quality samples for high content screening. A recent multiplexed addition to the Cellomics® HCS Reagent Kit line is the Synaptogenesis Kit. This kit enables simultaneous detection of neuronal populations, neurite, presynaptic vesicle, post-synaptic puncta and synapses using a fixed end-point assay based on immunofluorescence detection in cells.

"Traditionally, no reliable high throughput methods to measure multiple synaptogenetic factors have been available," said Michael Anhalt, Technical Product Manager at Thermo Scientific. Modulation of neurite and synaptic structures in neurons are closely related to the pathological process of neurological diseases. Mr Anhalt added: "This technology will greatly advance lead identification and validation in the study of neurobiology-based therapeutics. These diseases include: Alzheimer's, Parkinson's, autism, schizophrenia, anxiety/depression and addiction."

## Sensitive instrumentation for ion channel multiplexing

Ion channels are the third most-used target type in drug discovery according to HTBD's industry study. Most screening of ion channels has been done indirectly; however, recent innovations in patch-clamp technology have permitted automated and direct screening of ion channels. Dr James Costantin, IonWorks® Product Manager of MDS Analytical Technologies (www.moleculardevices.com), described how this technology can be used for multiplexing: "The IonWorks® Population Patch Clamp[1] (PPC) technology has allowed our customers to multiplex multiple ion channel targets at once in a single electrophysiology assay. Dale et al[2] describe different examples of multiplexed assays looking at two ion channel targets in a single assay." In one of the examples, a simultaneous screen and counter screen, the target Nav1.3 is multiplexed with the potentially undesirable target

**Table 1:** Needed improvements for multiplexing

| IMPROVEMENTS | NUMBER OF MENTIONS |
|---|---|
| Need more biochemical tools, appropriate antibodies, mass spec | 9 |
| Better signal-to-noise and more robust readouts, same quality data as single-read, eliminate false positives | 9 |
| Data management | 3 |
| Simultaneous screen and counter screen, selectivity | 3 |
| HCS needs to be faster and more robust | 3 |
| Multiplexing is too expensive | 3 |



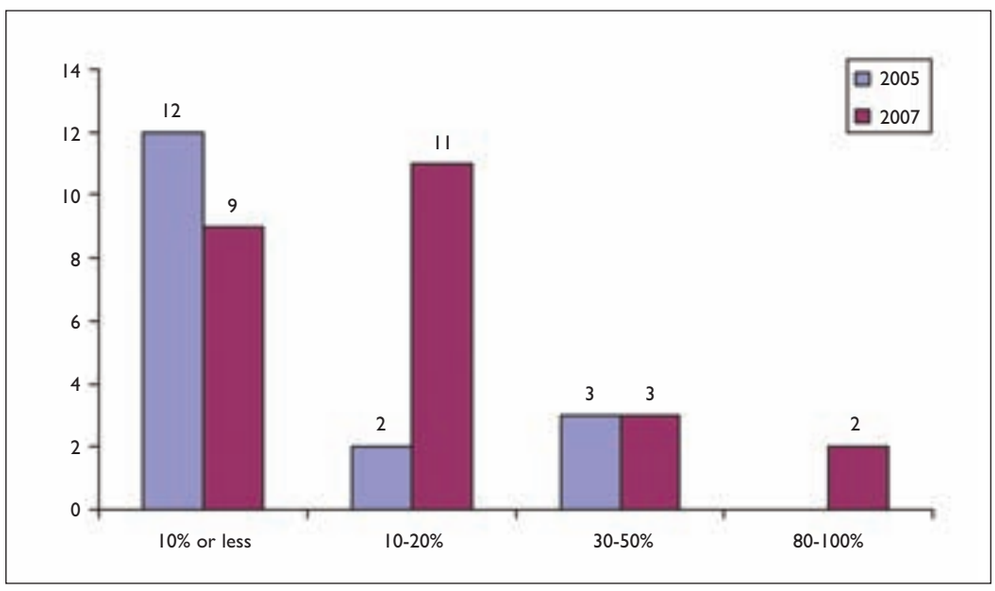
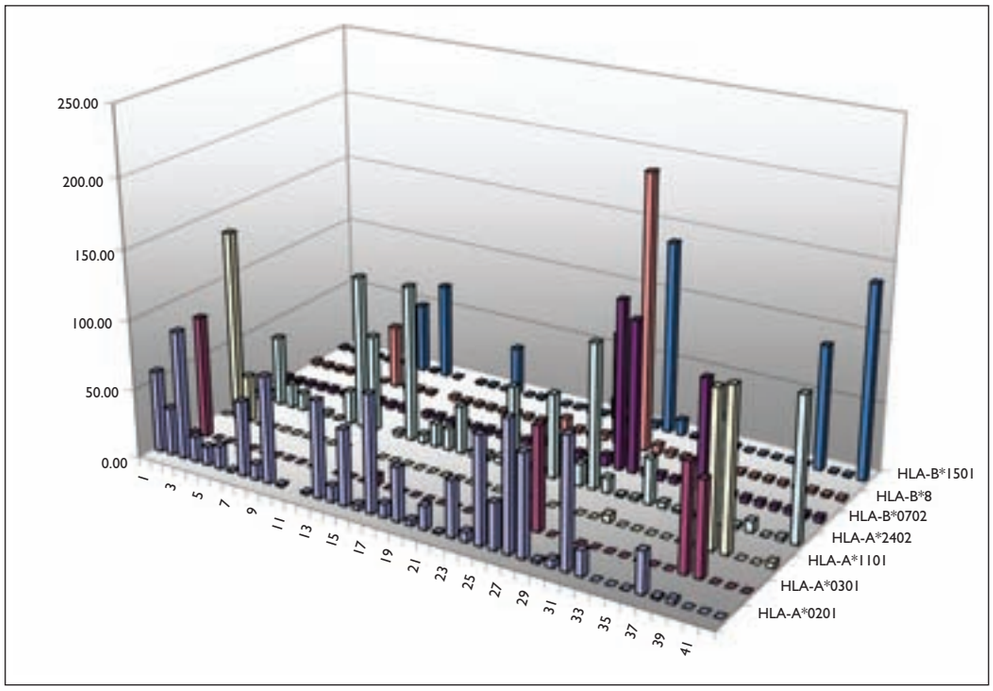



**Figure 1**
Percentage of assays performed in a multiplexed format




Results for MHC binding assay. Initial screening enables elimination of non-binders while generating data for ranking of binding candidates. *Image courtesy of Beckman Coulter, Inc*

Kv7.1. "Theoretically more ion channel targets, perhaps up to four, could be addressed with a carefully selected voltage protocol and a complementary set of ion channel targets," added Dr Costantin.

The best benefit to multiplexing using this PPC technology is the cost reduction through resource and time savings. Dr Costantin commented: "Direct electrophysiological (EP) assays for ion channel screens are expensive due to the cost of the substrate required to measure ionic currents in live cells. Direct EP assays are more desirable than fluorescent ion channel assays due to the near complete elimination of false positives and negatives. Direct EP assays have historically been done as a follow-up to a fluorescent (FLIPR) assay due to cost. Only since the introduction of PPC have direct EP screens been attempted in the 10,000 to 100,000 compound library range.

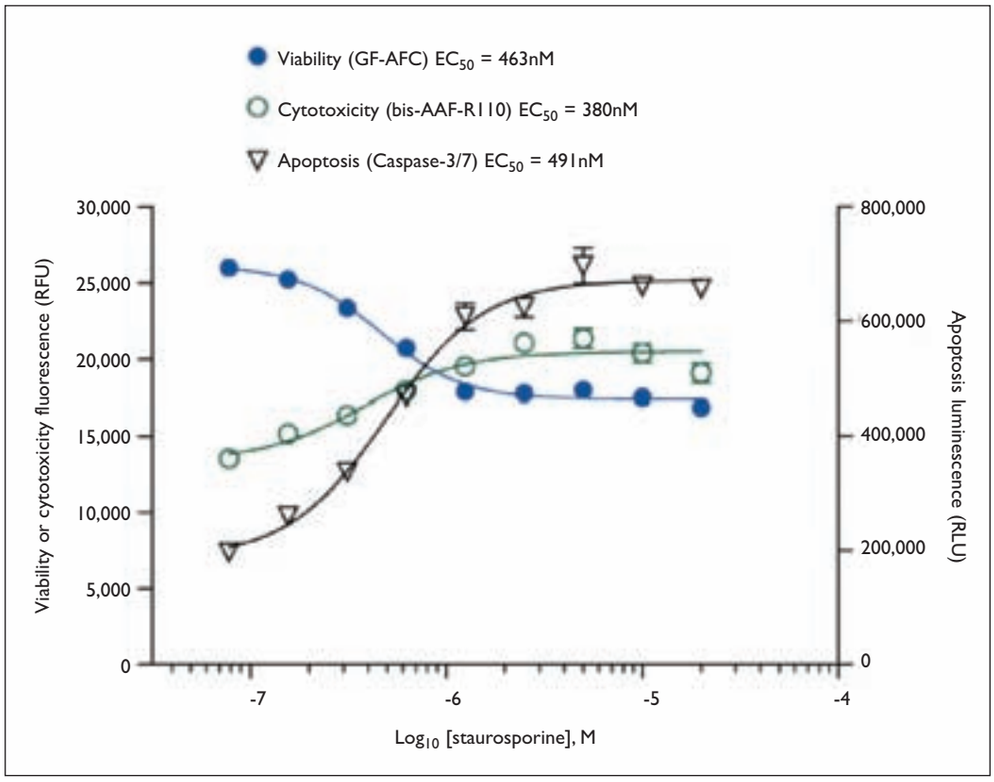
"The lower cost and higher throughput of multiplexing ion channel targets makes direct EP ion channel screens more attractive," Dr Costantin concluded.

## Custom assay optimisation

Beckman Coulter (www.beckmancoulter.com) offered its experience and expertise through the custom design of high content, multiplexed assays for drug discovery. "There are commercially available assays out there that give a multiplexed readout, but they are not optimised for a lab or research project and are not always reproducible," said Cassandra Vaughn, Global Strategic Market Manager for Flow Cytometry at Beckman Coulter. "Because of the biological complexity of these assays more and more clients are coming to us for help optimising high content screens."

"Our group is able to truly customise the assays so that they are standardised, automated and reproducible," explained Dr Wade E. Bolton, Vice President of Custom Biopharma Solutions at Beckman Coulter. "Standardisation is Beckman Coulter's strength. It comes from our diagnostics development where everything is optimised, validated, with well-written instructions that must meet strict FDA standards. We have worked with these assays for a long time so we have the experience to know which reagents will interfere and which will work together. Of course we always have to validate the assays to show there is no cross-talk, but that is what we do; we are a process driven company."

Dr Bolton added: "In addition, there are great economies of scale in multiplexing with 10 or more answers at the same time from one assay. For example, improved optics have led to better discernment of size, where we used to be able to detect two to five different bead sizes, now we can detect 50-100 differently sized antibody-tagged beads, and processing improvements allow us to detect 80,000 events per second. This is invaluable in areas where there is a huge amount of time-sensitive testing to be done, like vaccine testing."




Staurosporine is a known inducer of apoptosis. The ApoTox-Glo™ Triplex Assay measures three independent biomarkers to determine mechanism of toxicity: a live-cell protease activity for viability, a released dead-cell protease activity for cytotoxicity and caspase-3/7 activity for apoptosis. After Staurosporine treatment of Jurkat cells for six hours, the ApoTox-Glo™ Triplex Assay demonstrates a dose-dependent decrease in viability, increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis as the mechanism of cell death

## Multi reporter gene detector

Promega (www.promega.com) has created biochemical tools that offer a differential readout when combined in a high throughput triplex assay. Dr John Watson, Director of Cellular Analysis and Pharma/Biotech at Promega, described the company's approach: "We have developed the GloMax®-Multi platform into a fully integrated system for multiplexing luminescent and fluorescent assays used in small molecule screening." The GloMax®-Multi Detection System has independent luminescence, fluorescence and absorbance detection modules, providing light paths and detectors optimised for the best performance of each module.

"Our newest multiplexing technology enables the monitoring of apoptosis, cytotoxicity and viability in a single-well triplex assay. We are also looking to launch ApoLive-Glo® to monitor two of the biomarkers in the same well," said Dr Watson.

## Monitoring off-target effects

Drug discovery companies have pushed hard in the last decade to screen wide-ranging compound libraries to find useful small molecule drugs. Despite success identifying these candidates, they are too often failing in clinical trials. There is increased recognition that these candidates are failing because of off-target and cytotoxic effects and now high throughput screens are being utilised earlier in drug discovery to eliminate inappropriate candidates.

"Technology advancements in *in vitro* toxicology (IVT) assessment methods using automated cellular imaging techniques will have the biggest impact on decreasing late stage drop out," said Mr Anhalt of Thermo Scientific. "This technique offers the ability to measure multiple toxicity indicators in each cell of the exposed population. Combined cellular outputs are automatically compared to untreated populations producing a more sensitive, multi-faceted risk assessment tool for *in vitro* toxicity testing."

Promega's latest triplex assay monitoring apoptosis, cytotoxicity and cell viability was specifically designed to test toxicity. "Our expanded panel of assays uses *in vitro* data to help predict *in vivo* liabilities related to ADME properties (P450, MAO, PGP) and organ toxicity. They are typically used during the lead optimisation phase to help make better medicinal chemistry decisions," said Dr Watson.

IonWorks' PPC technology is also valuable for follow-up of targets and compounds first identified with indirect assays. Dr Costantin commented:

## References

1 Finkel, A et al. Population patch clamp improves data consistency and success rates in the measurement of ionic currents. J Biomol Screen, 2006. 11(5): p. 488-96.
2 Dale, TJ et al. Population patch clamp electrophysiology: a breakthrough technology for ion channel screening. Molecular Biosystems, 2007. 3: p. 714-722.

# Screen and profile kinases and ATPases with ease using a new bioluminescent ADP monitoring assay

By Dr Hicham Zegzouti and Dr Said Goueli

Because kinases and ATPases are validated drug targets, there is intense interest in robust technologies to monitor the activities of these enzymes in drug discovery programmes to develop novel therapeutics. Although several technologies exist, most of them are limited in their ability to address all the needs of kinase screening and profiling using a single platform. We recently developed a universal and homogeneous luminescence-based ADP detection assay, ADP-Glo™ Kinase Assay, that is applicable to all types of kinases, ATPases and other ADP producing enzymes.

The ADP-Glo Kinase Assay is universal and applicable to all classes of kinase substrates regardless of their nature with no prior modification (peptides, proteins, alcohols, lipids and sugars). The assay monitors ADP production and is directly proportional to light output via a coupled luciferase reaction. Sensitivity and linearity are hallmarks of the ADP assay. As demonstrated in panel A of **Figure 1**, there is a linear relationship between the luminescent signal and the amount of ADP in the reaction buffer at all ATP+ADP concentration series tested. In panel B, the high signal-to-background ratios at low ATP conversions illustrate the high sensitivity of the assay.

Most kinase researchers adopt radiometric assays as the most sensitive format, and such assays are often referred to as the 'gold standard' for kinase studies. The ADP-Glo Assay correlates with radioactive assays in terms of kinase inhibitor potency determination (data not shown).

The assay produces high signal-to-background ratios for all kinase enzymes tested to date. As evident in **Table 1**, only small amounts of enzyme per reaction are required to generate a signal-to-background ratio of 5 (SB5). In this data set, the percentage of ATP converted into ADP ranges between 2-5% for the enzymes listed.

**Table 1:** ADP-Glo Kinase Assay displays high signal strength at low ATP conversion

| ENZYME | SIGNAL-TO-BACKGROUND RATIO OF 5 (SB5) | % ATP TO ADP CONVERSION AT SB5 |
|---|---|---|
| EGFR | 0.3 ng | 2.2 |
| FAK | 0.6 ng | 2.5 |
| DNA-PK | 0.4 ng | 2.7 |
| IKKβ | 4.5 ng | 2.9 |
| PI3Kα | 0.9 ng | 1.6 |
| PI3 Kinase γ | 0.15 ng | 2.0 |
| Sphingosine Kinase 1 | 0.8 ng | 2.9 |
| Hexokinase | 0.1 ng | 4.6 |

## Conclusion

The ADP-Glo Kinase Assay offers many advantages as a platform technology for the screening and profiling of kinases, ATPases and other ADP producing enzymes. The lack of fluorescence interference, signal stability and ability to distinguish competitive from non-competitive ATP inhibitors render the assay ideal for primary and secondary screening; the universality and sensitivity render the assay suitable for profiling of lead compounds.

For more information on the new ADP-Glo Kinase Assay please see: www.promega.com/adpglo

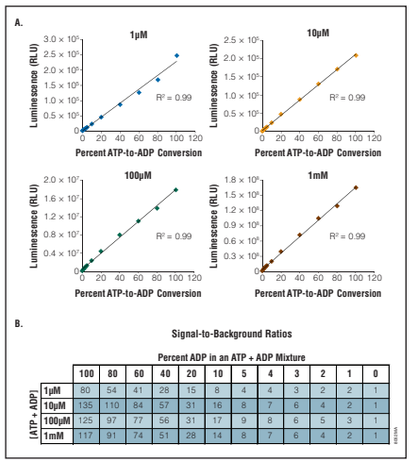



**Figure 1**
Sensitivity and linearity of the ADP-Glo Kinase Assay. Panel A: ADP-Glo Kinase Assay was performed on ATP-to-ADP conversion reactions for four different combinations of ATP+ADP. Panel B: Signal to-background ratios for varying amounts of ADP in a series of ATP+ADP mixtures

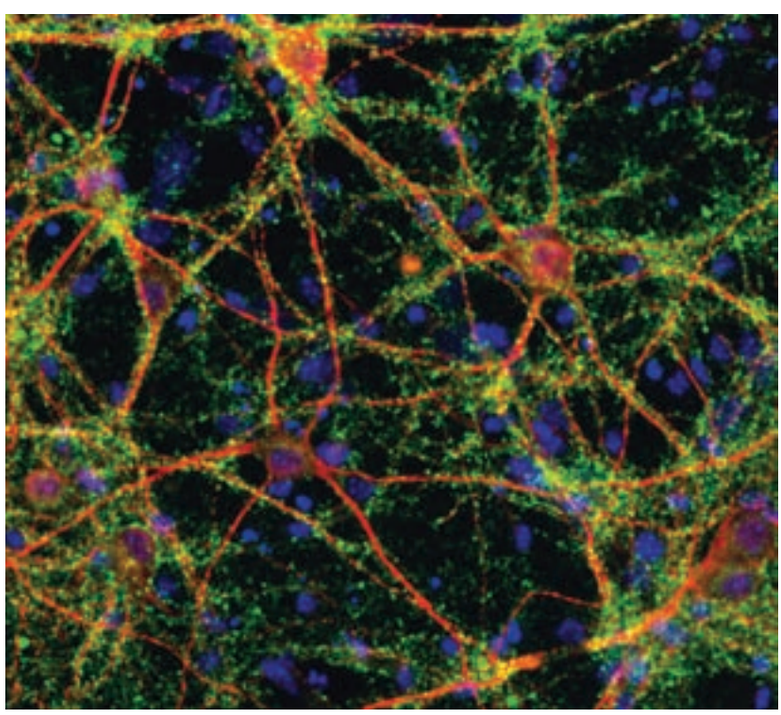
"Multiplexing enhances fluorescent follow-up assays by allowing the targets to be validated more quickly and allowing adverse affects on undesirable targets to be identified sooner."

Dr. Watson commented: "The key impact is to be able to drive better compounds into preclinical drug development by eliminating compounds with off target liabilities. This will speed up the lead validation phase and improve the leads prior to preclinical studies. The ability to get biologically relevant *in vitro* data has been the key to improved later stage productivity in small molecule drug discovery over the past couple of years."

*In vitro* toxicity testing has a cost benefit too, according to Mr Anhalt. "The costs of and the resistance against animal testing have increased the trend towards *in vitro* toxicity testing by both the pharmaceutical industry, to improve compound toxicity identification more efficiently and cheaply, as well as by non-pharmaceutical sectors. Cell-based *in vitro* toxicology testing offers economies of cost and speed to allow faster decisions prior to clinical studies."

## Multiplexing: an opportunity for improved discovery

Multiplexed assays provide a rich context for screening targets. Although these assays are complex, improvements in technology have made multiplexing more accessible to HTS labs.



Primary mouse cortical neurons were plated and maintained in a 96-well microplate for 14 days *in vitro*. The neurons were stained with Thermo Scientific's Synaptogenesis kit and visualised with Cellomics®ArrayScan Vti Instrument. All targets are superimposed in the image with pseudo-colours. DAPI stains nuclei (blue), Neuronal marker, MAP2 stains neurons (red) and Presynaptic marker, Synaptophysin stains presynaptic vesicles (green). Postsynaptic marker, PSD95 was not shown in this image

"I see a change happening in the way drug discovery is being done," said Dr Bolton. "There is an increase in high content screening which is important because we need to identify valid candidates early, instead of screening fast and dirty and then using a fail fast approach to eliminate candidates. It is better to identify the golden apple right away and move it forward." Dr Bolton continued: "Multiplexing is valuable because the more pieces of the puzzle we have in place simultaneously, the better the picture, as well as being able to identify the non-relevant pathways which ultimately leads to better medical efficacy. Assays can simultaneously look at a target, downstream events and parallel pathways. Screening this way can knock out ineffective or toxic compounds early on, decreasing later stage failures," Dr Bolton concluded.

"Multiplexing itself is the key to generating more biologically relevant *in vitro* data," said Dr Watson. "Because multiple biomarkers are being monitored in a single well, technical issues with things like the edge effect are controlled for. Plotting the data as a ratio of two analytes such as an apoptosis biomarker and a cytotoxicity indicator can reduce the coefficient of variability by up to three-fold. It also provides more information from a single well such as the potential mechanisms of toxicity." DDW

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