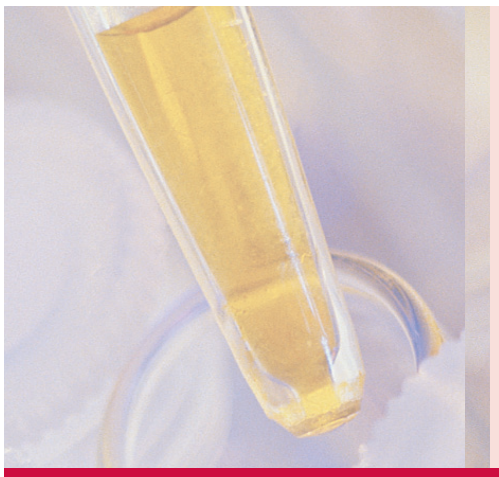


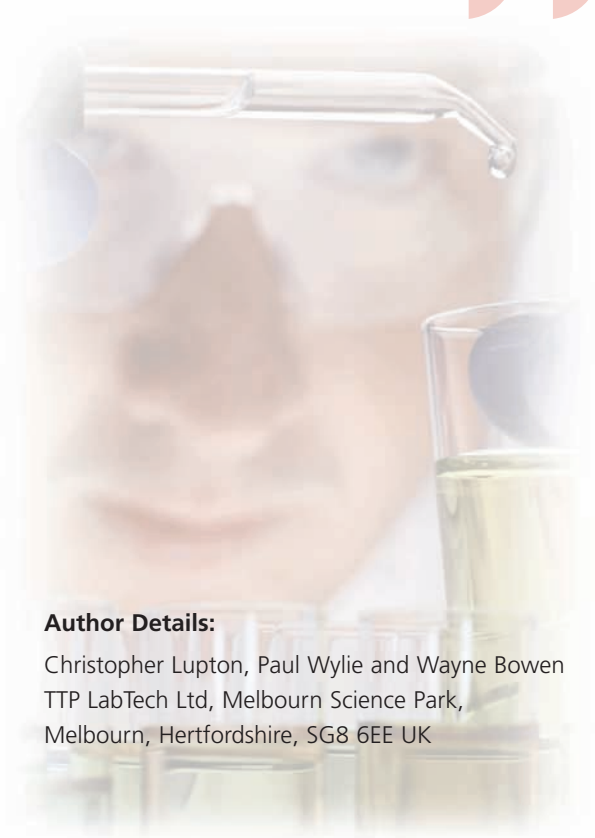
Product Focus

HIGH CONTENT ANALYSIS OF β-LACTAMASE REPORTER GENE ASSAYS USING MICROPLATE CYTOMETRY

Christopher Lupton, Paul Wylie and Wayne Bowen

High-throughput screening (HTS) plays a pivotal role in the identification of chemical leads for new therapeutic targets. Continuous technological advancement has made the screening aspect easier, but its expansion to include target identification and converting assay hits to qualified leads has created new challenges. A number of reporter gene assays have been widely employed for G-protein coupled receptor (GPCR) HTS since they can provide both drug affinity and functionality in a single screen. The application of high content analysis (HCA) technologies to such assays offers a new opportunity for improving their performance. Here we describe the use of microplate cytometry for β-lactamase reporter gene analysis and discuss its benefits in the context of improved quality of hit compound identification.

"MICROPLATE CYTOMETERS PROVIDE AN ALTERNATIVE METHOD FOR ANALYSIS AND REPORTING OF DATA..."



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G-protein coupled receptors represent the largest class of cell-surface receptors and the importance of their physiological roles makes them important pharmacological targets [1]. Consequently, a great deal of investment has been made in enabling the HTS of GPCRs. Traditionally, radioligand binding was widely used to determine the affinity of compounds at a target GPCR, but the method does not provide any functional information and is routinely applied in cell-free protocols. Increasingly, cell-based functional assays based on fluorescent labels are being used to maximise the amount of information obtained from each screening test. This shift aims to eliminate steps in the sequential and iterative hit-to-lead process.

Arguably, the most established cell-based screening assay platform for GPCRs is the FLIPR system (Molecular Devices, Sunnyvale, CA) which principally monitors release of calcium from intracellular stores using fluorescent reporter dyes [2]. FLIPR assays are reliable and useful for screening compound libraries, but analysis of receptors not coupled to calcium either requires construction of chimeric receptors or co-expression of genes encoding promiscuous G-proteins (e.g. Gα15, Gα16). An alternative approach is to use reporter gene technology whereby activation of a GPCR receptor at the cell membranes causes the expression of a specific gene – the 'report'. The resultant amount of reporter gene expression can be measured in a number of ways. One approach is to co-express a fluorescent protein with the target gene, whilst another is to monitor the catalytic activity of an expressed enzyme.

Expression of the β-lactamase enzyme has been very successfully applied for HTS of GPCRs due to the availability of commercial cell lines such as the GeneBLAzer® range from Invitrogen. These cells stably express the NFAT response element (for monitoring Ca^{2+} flux) and the cAMP response element (CRE) linked to the B-lactamase gene to provide a functional tool to identify GPCR activation [3]. GPCRs, regardless of Gα subunit coupling (Gs, Gq, Gi/Go), can be thus be monitored using β-lactamase gene expression. Key to the method development was the identification of the fluorescent β-lactamase substrates, CCF-2 and CCF-4.

These FRET-enabled substrates fluoresce green in the absence of β-lactamase reporter activity (FRET, Em ~530 nm), and blue (fluorescence, Em~460 nm) when cleaved thus providing a ratiometric readout *(Figure 1).*

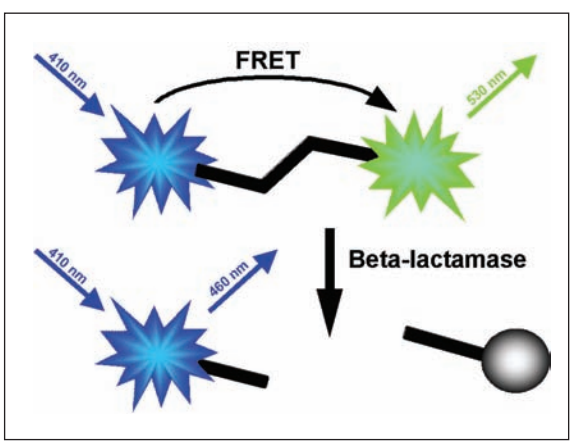

Figure 1. Ratiometric FRET - based substrates for β-lactamase activity

β-Lactamase activity has conventionally been analysed by bulk fluorescence readers [4] or flow cytometers [5], both of which require large numbers of cells (>10^4) for accurate analysis. Flow cytometry, although highly sensitive, has the added disadvantages of low throughput, and an inability to analyse adherent cell lines *in situ*. Microplate cytometers provide an alternative method for analysis and reporting of data, requiring as few as 100 cells per well in a 96 well plate.

For example, the Acumen Explorer microplate cytometer (TTP LabTech, Melbourn, UK) rapidly analyses any SBS format microplate in around 10 minutes. At the heart of the system is a proprietary, non-confocal optical system that performs whole well scanning, simultaneously collecting up to 4 colours. This removes the need for repeated collection of fluorescent emissions and decreases the overall read time in multiplex assays, thus giving a high screening potential comparable with throughputs of many primary screening campaigns [6].

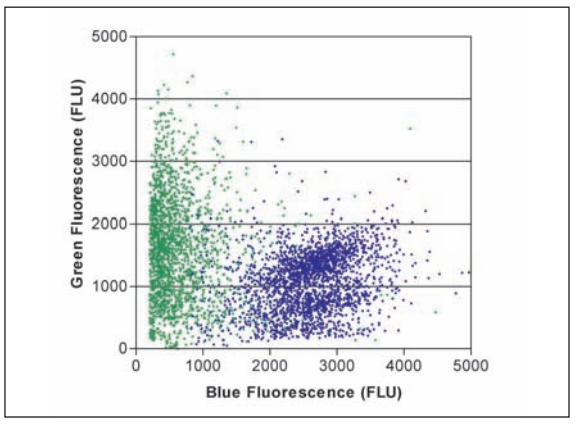

Figure 2. High content analysis of β-lactamase reporter gene expression

Many reporter gene assays can be quantified by microplate cytometry including those based on fluorescent proteins and enzyme substrates. The high content readout provided has many advantages. Firstly, the ability to define sub-populations within each well can increase the robustness of assays. This can be important for methods in which only a defined population of cells respond to the stimulant or possess the required amount of reporter gene activity, for example in assays using transiently-transfected cell cultures.

Secondly, use of the number of cells (including subpopulations) as a primary readout, rather than a fluorescent measurement, can overcome the significant heterogeneity often observed in the fluorescent intensity of active and inactive cells. Microplate cytometers, such as the Acumen Explorer equipped with 405 nm laser line, can simultaneously detect both the blue (β-lactamase expressing) and green (β-lactamase negative) regions of the spectrum to discriminate active from inactive cells.

Subsequent reporting of the number of active and inactive cells overcomes the scatter in the fluorescent intensity seen in the blue and green emissions. This routinely results in increased fold activation and assay robustness (Z') compared to bulk fluorescence measurements *(Figure 2).*

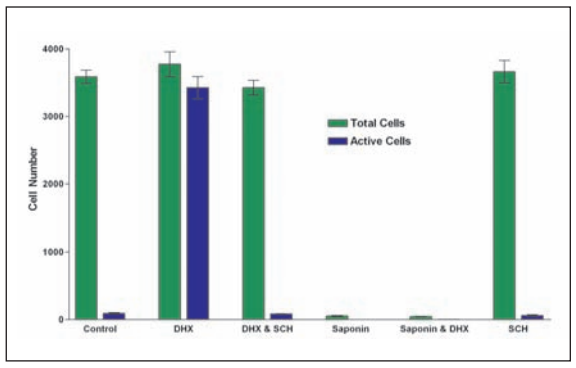

Figure 3. D1 dopamine receptor screening: Use of cell number a toxicity indicator

Furthermore, differentiating between cytotoxicity and antagonism can be difficult when analysing β-lactamase reporter gene assays using bulk readers, since both can result in the same reduction in total fluorescence signal. This increases the number of false positives returned by the assay when screening for GPCR antagonists. High content analysis reports the total number of cells in each well without the introduction of additional fluorescent markers, enabling cell loss associated with toxic compounds to be positively identified and differentiated from antagonism where the cell number remains constant *(Figure 3)*.

In conclusion, high content analysis has been shown to be directly applicable to β-lactamase reporter gene assays with little change to the standard methodology *(Figure 4)*. The reporting of ratiometric fluorescence data on a per cell basis dramatically reduces cell requirements from those used in bulk fluorescence assays without compromising assay performance. It also overcomes the significant heterogeneity observed in the amount of fluorescence per cell. The approach is also capable of distinguishing cytotoxicity from genuine receptor antagonism by reporting a total cell number without the use of additional fluorescent probes. Since microplate cytometers already offer rapid analysis of microplates compatible with primary screening campaigns, their combination with β-lactamase reporter gene technology equips the drug discovery community with a powerful new partnership for use in GPCR drug discovery.

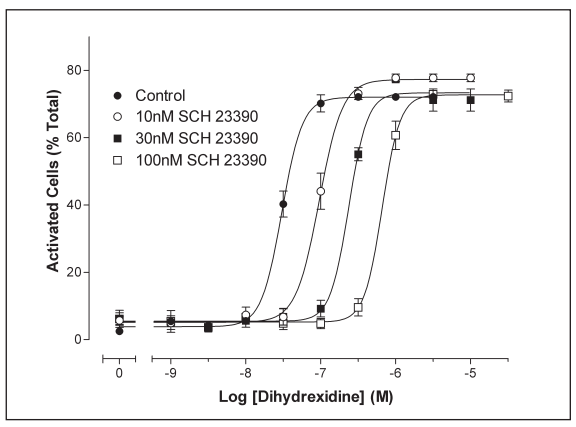

Figure 4. Antagonism of D1 dopamine receptors by SCH-23390

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