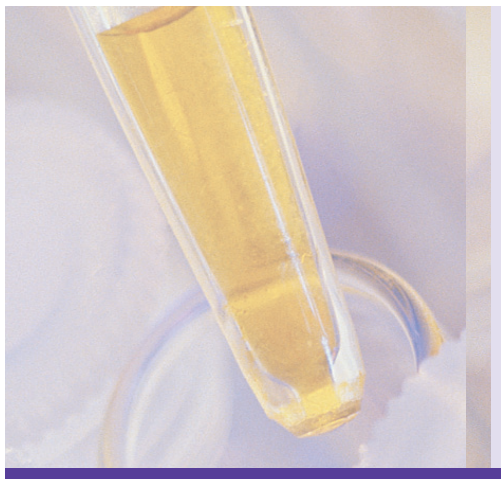

# Laboratory Product Focus

## SCREEN FOR CYTOCHROME P450 ACTIVITY WITH IMPROVED SENSITIVITY AND DECREASED INTERFERENCE

Dr Desdemona De Souza, Product Manager, Promega UK Ltd

***Drug discovery is often like looking for a needle in a haystack. Finding the one compound with the specific properties to make a useful drug, amongst millions of compounds, is a lengthy and expensive process. In vitro assays that predict the in vivo behaviour of compounds are becoming very important to the drug discovery industry, as these assays enable good drugs to reach the patient faster and save money by eliminating poor candidates earlier in the process.***

***"...INTERACTIONS ARE CAREFULLY INVESTIGATED BY PHARMACEUTICAL COMPANIES EARLY IN THE DISCOVERY PROCESS WHERE THE "FAIL EARLY – FAIL CHEAPLY" PRINCIPAL IS KEY."***

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Cytochrome P450 enzymes are central to drug metabolism, catalysing the oxidative metabolism of a vast array of hydrophobic chemicals [1]. P450 enzymes influence both drug bioavailability and clearance and many adverse drug-drug interactions (DDIs) are attributed to altered P450 activities [2,3]. In these situations, a first drug might inhibit a P450 that metabolises a second co-administered drug so the first drug slows clearance of the second, causing it to accumulate to a toxic level. P450-dependent DDIs also occur when a first drug induces expression of a P450 gene, leading to increased P450 activity, accelerated clearance and reduced efficacy of a second drug. Due to the central role P450 enzymes play in drug disposition, P450-drug interactions are carefully investigated by pharmaceutical companies early in the discovery process where the "fail early – fail cheaply" principal is key.

There are 57 functional cytochrome P450 genes in the human genome [4], but enzymes encoded by only five genes account for most P450-dependent drug metabolism [5]. Most drug metabolism takes place in the liver, and CYP3A4 is the major hepatic P450 enzyme accounting for about half of all known drug biotransformations. CYP2D6 is second to CYP3A4 in terms of drug biotransformation activities, and CYP2C9, CYP1A2 and CYP2C19 also oxidise many drugs. (Although, other P450 enzymes metabolise drugs, these five enzymes are responsible for most drug metabolism.)

Small-molecule drug discovery involves screening large chemical libraries against primary drug targets. P450 enzyme screens are essential for prioritising hits. For this purpose, a rapid, high throughput method is required. A common approach is to use fluorescent substrates. However, there are disadvantages with this method, which have been addressed by the design of a bioluminescent P450 assay.

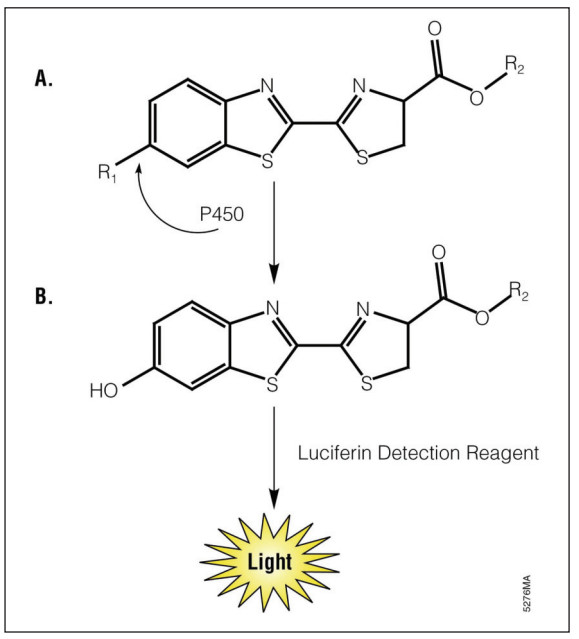


*Figure 1.* ***P450-Glo™ reaction scheme****.*
*Compound A represents the P450-Glo™ Substrates and compound B, the luminogenic reaction products that generate light with P450-Glo™ Luciferin Detection Reagent. P450 enzyme selectivity for P450-Glo™ Substrates is dependent on R1 and R2. CYP1A2 substrate: R1 = CH3O, R2 = H; CYP2C9 substrate: R1 = H, R2 = H; CYP2C19 substrate: R1 = H, R2 = C2H4OH; CYP2D6 substrate: R1 = CH3O, R2 = C2H4OH; CYP3A4 substrate: R1 = O-benzyl, R2 = H.*

### LUMINESCENT ASSAY PRINCIPLE

In nature, bioluminescence is produced in an ATP dependant reaction by the action of the luciferase enzyme on its substrate luciferin. This reaction has previously been harnessed for genetic reporter assays and cell viability determination. For P450 assays, this reaction has been taken a step further. Promega's P450-Glo™ system uses a chemically modified pro-luciferin substrate, which is cleaved by P450 enzymes to give luciferin. The luciferin then reacts with luciferase to give light. There is a direct correlation between luciferin product and light output, so luminescence is used as a measure of P450 activity (*Figure 1*). Test compounds that modulate P450 enzyme activity change the amount of luciferin product, and this is reflected in the luminescent output of the reaction. The most common effect of test compounds that influence P450 enzyme activity is inhibition, though activators are occasionally observed.

### MINIMISING BACKGROUND TO ACHIEVE GREATER SENSITIVITY

The bioluminescent approach of the P450-Glo™ Assays has fundamental advantages over fluorescence. Detection of fluorescent signals requires excitation of the fluorophore with a laser and because the wavelengths of the excitation photons and the emission photons typically overlap there is a practical limit to the sensitivity that can be achieved. The excitation light creates high-background signals that that can hide changes in P450 activity. Since bioluminescence does not require excitation, background signals are lower and assays are far more sensitive. In addition, fluorescence suffers from overlap between the fluorescent spectra of the probe substrates and fluorescent analytes or other assay components (e.g. NADPH). These artifacts are eliminated with the bioluminescent approach. A further advantage is found in the improved water solubility of the P450-Glo™ luminogenic substrates compared to typical fluorescent substrates. Though test compounds may require the addition of some organic solvent, little or no additional solvent is introduced with the P450-Glo™ Substrates. Luminescent assays also show improved linearity with respect to enzyme concentration, a feature that is probably influenced by the water solubility of the luminogenic substrates (*Figure 2*).

### SELECTIVITY OF LUMINOGENIC SUBSTRATES

The P450 luminogenic assay systems currently available are configured around six unique derivatives of D-luciferin. In terms of selectivity, Luciferin-H (deoxyluciferin) is extremely selective for CYP2C9 and so can be used with liver microsomes and hepatocytes (*Figure 3*). Luciferin-BE reacts with three P450 enzymes, CYP3A4, -3A7 and -4F12 (*Figure 3*). As expression of CYP3A7 and -4F12 is minimal in adult liver, luciferin-BE can be used in microsomes and hepatocytes to look at CYP3A4 activity. Luciferin-CEE (luciferin 6'cholortheyl ether) also reacts with three enzymes, CYP1A1, -1B1 and -3A7 (*Figure 3*). Again this substrate is selective by default for just one enzyme (CYP1A1), as CYP1B1 and -3A7 are minimally expressed in adult liver.

### OBTAINING ACCURATE IC50 VALUES

Luminescent assay technology is an ideal tool for screening compound libraries. A single concentration, typically 10µM, of many compounds is screened against one or more P450 enzymes, or a range of concentrations is screened to determine the dose dependency of an effect (*Figure 4*). IC50 values measured using the luminescent assays agree with published values from fluorescent or non-optical methods [7,8].

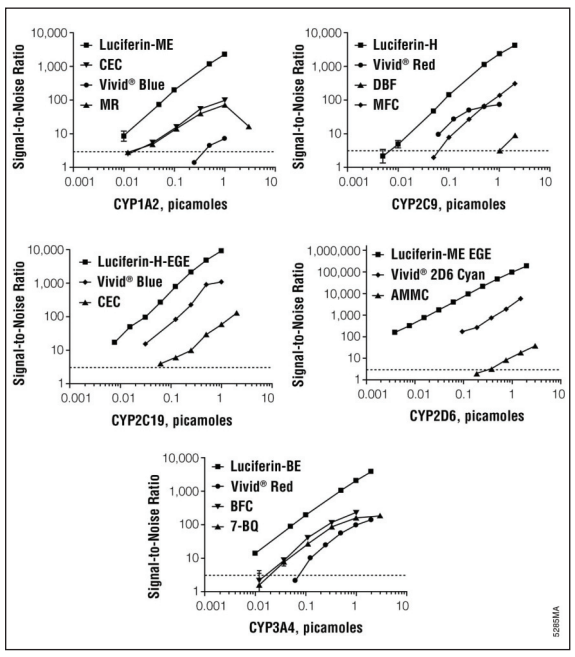


*Figure 2.* ***P450 assay sensitivity***.
*P450 assays were performed at 37ºC with recombinant P450 microsome fractions from an insect cell expression system (Supersomes™ System). P450-Glo™ Assays were performed as described by Promega and fluorescent assays as described by Invitrogen Life Technologies (Vivid® substrates) or BD/Gentest (MR=methoxyresorufin, DBF=dibenzylfluorescein, CEC=3-Cyano-7-ethoxycoumarin, AMMC=3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin, 7-BQ=7-benzyloxyquinoline, BFC=7-Benzyloxy-4-(trifluoromethyl)-coumarin, MFC = 7-methoxy-4-fluoromethylcoumarin). To calculate signal-to-noise ratios, relative luminescent or fluorescent measurements were made with a plate reading luminometer or fluorometer, respectively, from samples plus P450 and minus P450 controls (background). Average background measurements were subtracted from plus P450 measurements to give signals, and signals were divided by the noise (noise = the standard deviation of the background) [6].*

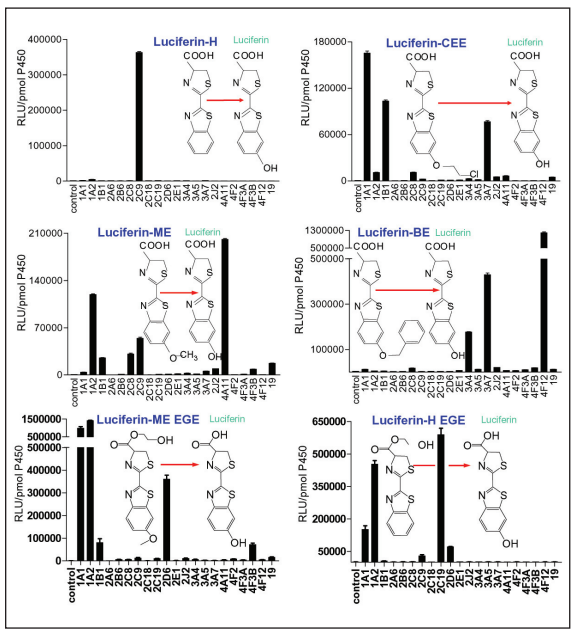


*Figure 3.* ***Selectivity of P450-Glo™ substrates***.
*Twenty-one human P450 enzymes were assayed with six luminogenic substrates (luciferin-CEE, luciferin-ME, luciferin-H, luciferin-BE, luciferin-H-EGE and luciferin-ME-EGE). The P450 enzymes were recombinant forms expressed in insect cells and prepared as microsomal fractions (from Discovery Labware and Promega). Control reactions use an insect cell microsomal fraction devoid of P450 activity. Each substrate was assayed at 50 µM in a 50 µl volume with 1 pmol of each CYP enzyme at 37°C in a white 96-well luminometer plate. Reactions were initiated and sustained by adding an NADPH regeneration mixture consisting of NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and Mg2+. After 30 min, reactions were stopped by the addition of LDR and luminescence was read in RLU 20 min later on a multiwell plate-reading luminometer (GloMax™, Promega).*

*LDR: Luciferin detection reagent; Luciferin-BE: Luciferin 6'benzyl ether: Luciferin-CEE: Luciferin 6'chloroethyl ether; Luciferin-H: Deoxyluciferin; Luciferin-H EGE: Ethylene glycol ester of deoxyluciferin; Luciferin-ME: Luciferin 6'methyl ether; Luciferin-ME-EGE: Ethylene glycol ester of luciferin 6'methyl ether; RLU: Relative light units.*

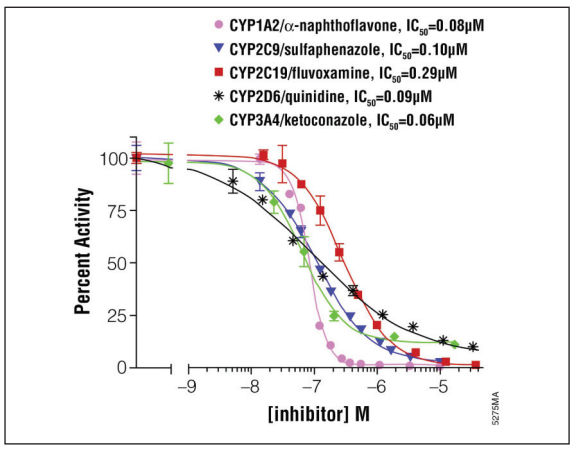


*Figure 4.* ***Using P450-Glo™ Assays to measure P450 inhibition***.
*Recombinant P450s (Supersomes™ System) were assayed at the Km concentrations of their respective substrates and the indicated concentrations of inhibitors following the protocols described by Promega. Curve fits and IC50 calculations were performed with the GraphPad Prism® program.*

## ALTERNATIVE TECHNOLOGIES

Alternatives to luminescent P450 assays can be classed as either optical or non-optical methods. The approach depends primarily on the measurable properties of the P450 reaction product (*Table 1*). The main optical alternatives are fluorogenic assays, which like luminogenic assays measure photons emitted from an excited state molecule. Non-optical assays may be radioactive or non-radioactive, and require a separation step.

The non-optical P450 assay approach of choice uses unlabelled substrates with HPLC for sample separation and mass spectrometry for product detection (liquid chromatography mass spectrometry [LC-MS]). This approach has an advantage of both sensitivity and versatility for both in vitro and cell-based assays. Substrate selection is not limited by the need for an optical property in the reaction product and a cocktail of P450 selective substrates can be incubated simultaneously with liver microsomes, where multiple P450s are coexpressed or with mixtures of recombinant P450s [9,10]. LC-MS cell-based assays analyse culture medium, cell lysates or reactions with microsomes prepared from cells. In addition to screening applications, LC-MS is used for metabolite identification, which is only possible to a very limited extent with optical assays. Although it has many advantages LC-MS is labour intensive, time consuming and uses expensive instrumentation.

Radioactive P450 assays with labelled substrates produce labelled products that are typically separated by HPLC or some other mode of solid-phase extraction and detected radiometrically. The possibility of false hits due to optical probe interference or luciferase inhibition is eliminated in a radiometric approach [11]. In addition, these assays have a selectivity and sensitivity advantage, but due to the HPLC step, throughput is limited. Hence, radioactive assays could not be used in a high throughput scenario.

To maximise assay throughput without radioactivity, probe substrates that yield a fluorescent product after metabolism by a P450 are commonly used. The fluorogenic approach has the advantage of monitoring real-time reaction product accumulation; fluorescence from a reaction is simply read and re-read as the time course progresses. However, the convenience of fluorescence-based assays is counterbalanced by some important limitations. High background fluorescence limits the working range and sensitivity of the assays. In addition, the fluorescent properties of certain chemical molecules may overlap with those of the probe and confound analysis. In addition, the aqueous solubility of many fluorescent P450 substrates is poor. This may necessitate a need to include an excessive amount of organic solvent in reactions and can compromise use for quantitative analyses. Some fluorescent P450 assays with intact cells have been reported, but there has been limited success with this approach, probably due to limited cell permeability, intracellular distribution of the substrates or poor selectivity [12].

## SUMMARY

The luminescent technology offers a significant leap forward for the study of this important family of enzymes. By adapting the fundamental advantages of bioluminescence, researchers can move beyond many of the limiting features of traditional methods.

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*Table 1.* ***P450 enzyme assay methods***

| Method | HPLC based | Radioactive | Fluorescent | Luminescent derivative |
|---|---|---|---|---|
| P450 substrate | Non-optical | Radiolabelled | Fluorogenic | Lucferin |
| P450 products | Non-optical | Radiolabelled | Fluorescent | D-luciferin |
| Detection | MS, UV, absorbance | Scintillation counter | Fluorometer | Luminometer |
| Recombinant P450s | Yes | Yes | Yes | Yes |
| Liver microsomes | Yes | Yes | Some | Some |
| Intact cells | Yes | Yes | Few | Some |
| Metabolite identification | Yes | Some | No | No |
| Sensitivity | High | High | Moderate | High |
| Fluorescent interference | No | No | Yes | No |
| Homogenous | No | Some | Yes | Yes |
| Throughput | Low to medium | Low to high | High | High |

D-luciferin; [4*S*]-4,5-dihydro-2-[6'-hydroxy-2'-benzothiazolyl]-4-thiazolecarboxylic acid; MS: Mass spectrometry; UV: Ultraviolet.