SCREEN FOR CYTOCHROME P450 ACTIVITY USING A LUMINESCENT ASSAY

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Introduction

Cytochrome P450 enzymes are the major catalysts for the oxidative metabolism of a vast array of hydrophobic chemicals (1). Some P450 substrates are endogenous to organisms, and others are exogenous chemicals known as xenobiotics. Small-molecule therapeutic drugs are an important class of xenobiotics. Metabolism of drugs by P450 enzymes influences drug clearance, toxicity, activation and in some cases, adverse interactions with other drugs (2). Compounds that are turned over rapidly or that are converted to toxic products by P450 enzymes may be poor drug candidates. Furthermore P450-based drug-drug interactions occur when the influence of one drug on a P450 enzyme changes the disposition of a second co-administered drug. For example, the breakdown of a first drug by a P450 enzyme will be slowed if it is co-administered with a second drug that inhibits the P450 enzyme. This might cause the first drug to accumulate to a toxic level. In another example, a drug that induces expression of a gene encoding a P450 enzyme responsible for the clearance of the second drug will speed clearance of the second drug and reduce efficacy. Because of the central role P450 enzymes play in drug disposition, P450-drug interactions are carefully investigated.

P450-Glo™ Assays enable researchers to move beyond many limiting features of traditional methods.

The human genome contains 57 putative functional cytochrome P450 genes (3), and enzymes encoded by only five of them account for most P450-dependent drug metabolism (4). The liver is the major site for drug metabolism, 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 oxidize many drugs. Other P450 enzymes metabolize drugs, but CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2C19 enzymes are responsible for most drug metabolism.

Small-molecule drug discovery involves screening large chemical libraries against primary drug targets, and P450 enzyme screens are essential for prioritizing hits. Entire libraries may be screened against P450 enzymes so that each compound can be annotated with information about P450 enzyme interactions *a priori*, or hits from a primary screen may be subjected to a secondary screen against P450 enzymes.

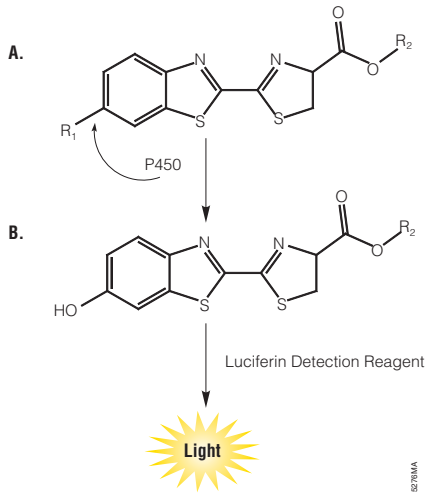

Figure 1. P450-Glo™ reaction scheme. Compound A represents the P450-Glo™ Substrates and compound B, the luminogenic reaction products that generate light with P450-Glo™ Luciferin Detection Reagent. P450 enzyme selectivity for P450-Glo™ Substrates is dependent on R_1 and R_2. CYP1A2 substrate: $R_1 = CH_3O$, $R_2 = H$; CYP2C9 substrate: $R_1 = H$, $R_2 = H$; CYP2C19 substrate: $R_1 = H$, $R_2 = C_2H_4OH$; CYP2D6 substrate: $R_1 = CH_3O$, $R_2 = C_2H_4OH$; CYP3A4 substrate: R_1 = O-benzyl, $R_2 = H$.

For both approaches a rapid, high-throughput method is needed. A common approach is to use a substrate that yields a fluorescent product after metabolism by a P450 enzyme. Test compounds that modulate P450 activity are identified as those that influence fluorescent product accumulation. This approach presents some disadvantages, which we have addressed in the design of the bioluminescent P450-Glo™ Assays.

Assess P450 Activity with a Luminescent Assay

P450-Glo™ Assays take advantage of the light-generating reaction of firefly luciferase with its substrate luciferin. A derivative of luciferin that is a P450 enzyme substrate but is not a substrate for luciferase is provided. In a first reaction, a P450 enzyme converts the derivative to a luciferin product that is detected in a second reaction with a luciferin detection reagent that generates luminescence (Figure 1). There is a direct correlation between the amount of luciferin product and the light output of the luciferase reaction, so luminescence is used as a measure of P450 enzyme activity. 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.

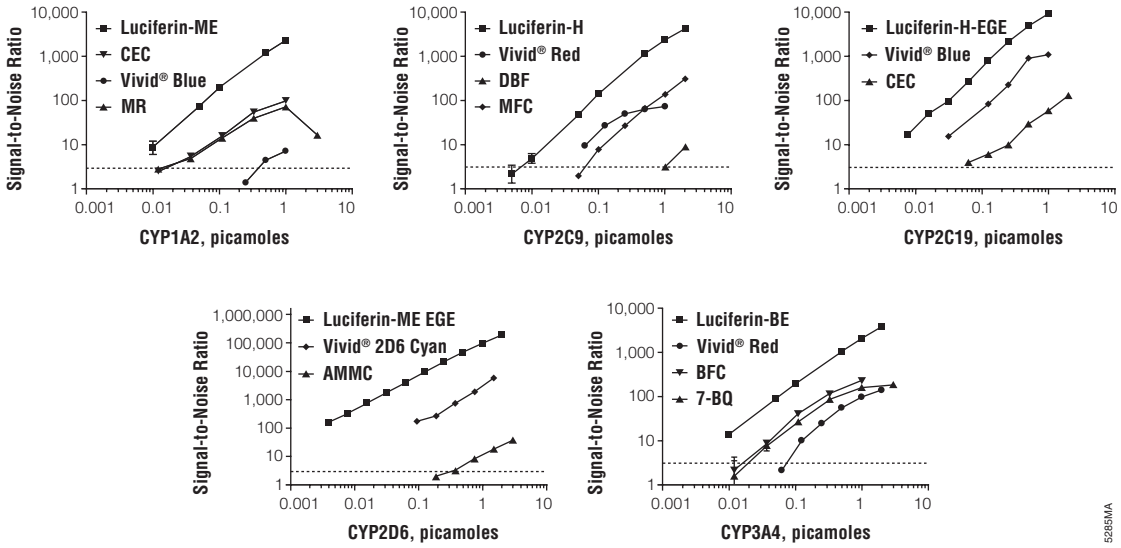

Figure 2. P450 assay sensitivity. P450 assays were performed at 37ºC with recombinant P450 microsome fractions from an insect cell expression system (Supersomes™ System). P450-Glo™ Assays were performed as described in Technical Bulletins #TB325 and #TB340, and fluorescent assays as described by Invitrogen 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). **Note:** P450-Glo™ Assays for CYP2C19 and CYP2D6 isoforms are currently under development.

Minimize Background and Achieve Greater Sensitivity

The bioluminescent approach of P450-Glo™ Assays has certain fundamental advantages over fluorescence. For example, the excitation light required for fluorescent methods can create high-background signals that limit assay sensitivity. Bioluminescence does not require an excitation light, so background signals are lower and assays more sensitive. Also, misleading results due to overlap between fluorescent spectra of the probe substrates and fluorescent analytes or other assay components (e.g., NADPH) are eliminated with the bioluminescent approach. An additional advantage is foundin the improved water solubility of the P450-Glo™ luminogenic substrates compared to typical fluorescent substrates. Though properties of test compounds may require adding some organic solvent to reactions, little or no additional solvent is introduced with P450-Glo™ Substrates. Luminescent assays also show improved linearity with respect to enzyme concentration, a feature that is likely influenced by the water solubility of the luminogenic substrates (Figure 2).

The sensitivity of P450-Glo™ CYP1A2, CYP2C9, CYP2C19(1), CYP2D6(1) and CYP3A4 Assays relative to fluorescent assays for the same enzymes was examined (Figure 2). For these experiments, a range of concentrations of recombinant human P450s from an insect cell expression system were assayed. Signal-to-noise ratios from the P450-Glo™ Assays and the corresponding fluorescent assays were plotted on the same graph to facilitate comparisons. The full range of enzyme concentrations tested is shown except for Vivid® 2D6 Cyan, Vivid® 2C9 Red and Vivid® 2C19 Blue, where enzyme-dependent changes in signal were not observed at less than the lowest enzyme concentration shown. In each case, the P450-Glo™ Assay was more sensitive than fluorescence. Thus in P450-Glo™ Assays, less enzyme is required. This leads to lower nonspecific binding of analytes and substrates to components of the enzyme preparation (5). This nonspecific binding reduces effective concentrations and leads to over estimation of an inhibitor's K_i and under estimation of in vivo interactions.

The large signal-to-noise ratios from P450-Glo™ Assays (Figure 2) result in robust, high-quality assays. Z´-factor is the commonly accepted numerical assessment of assay quality. A perfect assay has a Z´-factor value equal to 1.0, and assays with a Z´-factor value greater than 0.5 are usually viewed as acceptable for HTS (6). Z´-factor values for P450-Glo™ Assays are typically greater than 0.8.

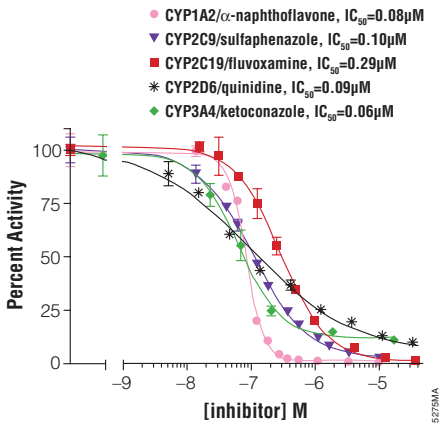

Figure 3. Using P450-Glo™ Assays to measure P450 inhibition. Recombinant P450s (Supersomes™ System) were assayed at the K_m concentrations of their respective substrates and the indicated concentrations of inhibitors following the protocols described in Technical Bulletins #TB325 and #TB340. Curve fits and IC_{50} calculations were performed with the GraphPad Prism® program.

Obtain Accurate IC_{50} Values

The P450-Glo™ 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 3). IC_{50} values measured using the P450-Glo™ Assay are in good agreement with published values from assays that used fluorescent or nonoptical methods (7,8). To perform a screen, compounds are dispensed into 96- or 384-well plates with a P450 reaction mix containing reagents and an appropriate luminogenic P450 substrate. At the end of the P450 reaction incubation, an equal volume of a luciferin detection reagent is added. This reagent stops the P450 reaction and simultaneously initiates a glow-style luminescent reaction with a half-life of greater than two hours. The magnitude of the light signal is dependent on the product generated by the P450 reaction.

Summary

The P450-Glo™ Technology is a leading-edge technology that 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. This simple-to-use robust assay is highly sensitive and amenable to automation. These features, combined with the low incidence of false positives resulting from compound interference, make the P450-Glo™ Assay the method of choice for HTS of cytochrome P450 enzymes. ■

References

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Protocols

P450-Glo™ Assay Systems Technical Bulletin #TB325 (www.promega.com/tbs/tb325/tb325.html)

P450-Glo™ Screening Systems Technical Bulletin #TB340 (www.promega.com/tbs/tb340/tb340.html)

Ordering Information

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	50ml*	V8752
P450-Glo™ CYP1B1 Assay	50ml*	V8762
P450-Glo™ CYP1A2 Assay	50ml*	V8772
P450-Glo™ CYP2C8 Assay	50ml*	V8782
P450-Glo™ CYP2C9 Assay	50ml*	V8792
P450-Glo™ CYP3A4 Assay	50ml*	V8802
P450-Glo™ CYP3A7 Assay	50ml*	V8812
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800

*Products available in additional sizes.

[1]P450-Glo™ Assays for isoforms for CYP2D6 and CYP2C19 are currently under development.