SCREEN FOR CYP450 INHIBITORS USING P450-GLO™ LUMINESCENT CYTOCHROME P450 ASSAYS

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The new P450-Glo™ CYP450 Assays provide a homogeneous, luminescent method for measuring cytochrome P450 activity. The assays are designed for measuring the activities of P450s from recombinant and native sources and for testing the effects of analytes such as drugs and new chemical entities on P450 activities in a multiwell format. P450-Glo™ Luminescent Assays exhibit exquisite sensitivity, low background signals and broad dynamic range.

Introduction

The cytochromes P450 (CYP450) are a superfamily of enzymes that catalyze the oxidative metabolism of a diverse set of hydrophobic chemicals, including most therapeutic drugs (1). CYP450-mediated metabolism influences clearance rates of drugs, their toxicity and their interactions with co-administered drugs. In the arena of drug discovery, researchers need to determine how new drug entities are metabolized by CYP450s and to what extent they may alter CYP450 activity. Some of these determinations can be achieved by using known CYP450 substrates as probes (2). Useful probe substrates are compounds that change in a measurable way when they react with a CYP450. If the reactivity of the probe is altered in the presence of a drug, researchers can conclude that the drug indeed has an impact on CYP450 activity. For example, CYP450 inhibitors are readily identified if they reduce the reactivity of a CYP450 with a probe substrate.

Assay Principle

P450-Glo™ Assays[a,b] employ luminogenic CYP450 probe substrates that are derivatives of beetle luciferin, a substrate for luciferase enzymes. The derivatives are not substrates for luciferase but are converted by P450s to luciferin, which in turn reacts with luciferase to produce an amount of light that is directly proportional to the activity of the P450 (Figure 1). A luminogenic substrate is incubated with an active CYP450 preparation (Figure 2). The CYP450 activity is stopped and luciferin detected by adding a luciferin detection reagent. The P450-Glo™ CYP450 Assays use Ultra-Glo™ Luciferase, a recombinant, stable luciferase in a proprietary buffer system, that generates a "glow-type" luminescent signal. The half-life of the luminescent output is greater than two hours, eliminating the need for luminometers with injectors and allowing batch plate processing. The formulation also minimizes the incidence of false positives due to inhibition of luciferase by analytes.

P450-Glo™ Luminescent Assays overcome many of the limitations of commonly used fluorescence-based and nonoptical CYP450 assays. Advantages of this assay system include:

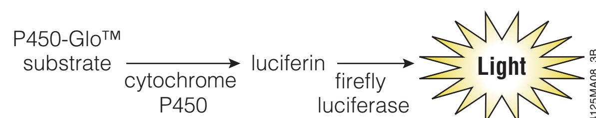


Figure 1. The P450-Glo™ Assay reaction scheme. CYP450 enzymes convert the P450-Glo™ luminogenic substrates to luciferin, a substrate for firefly luciferase. Luciferase uses luciferin to produce light.

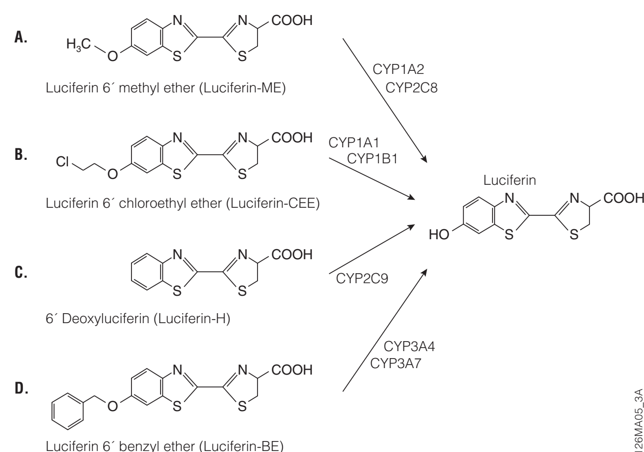


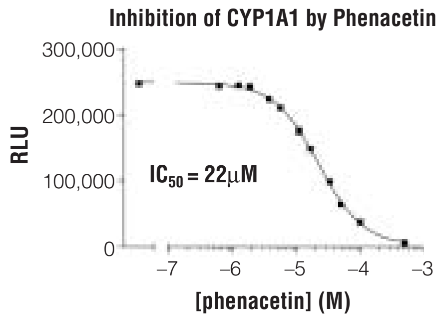
Figure 2. P450-Glo™ luminogenic substrates are converted to luciferin by CYP450s. Reaction A. The substrate for CYP1A2 and CYP2C8 is Luciferin-ME. **Reaction B.** The substrate for CYP1A1 and CYP1B1 is Luciferin-CEE. **Reaction C.** The substrate for CYP2C9 is Luciferin-H. **Reaction D.** The substrate for CYP3A4 and CYP3A7 is Luciferin-BE.

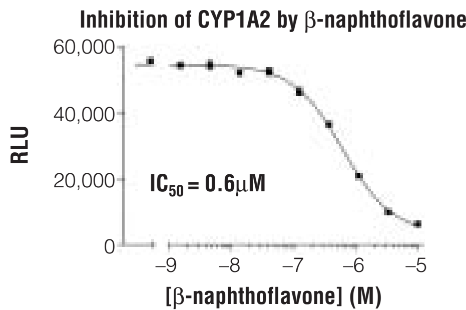
No Fluorescence Interference: Because the assays are luminescent, there are no fluorescent excitation and emission overlaps between analytes, NADPH and cytochrome P450 substrates to confound analysis.

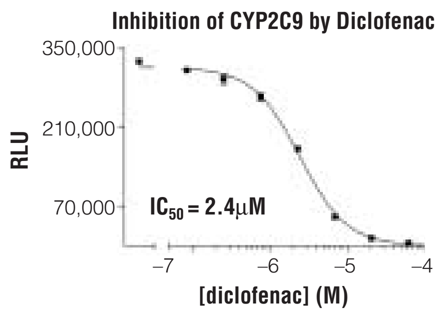
Speed: The luminescent format eliminates the need for time-consuming analyses such as HPLC.

Simple Method: These homogeneous assays are amenable to high-throughput screening in multiwell plates by manual or automated methods.

Greater Sensitivity: Low background levels and a large dynamic range result in sensitive assays that require less CYP450 than typical HPLC or fluorometric methods.







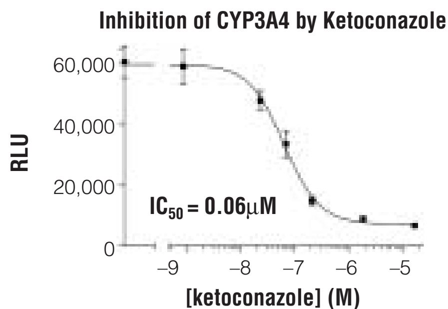
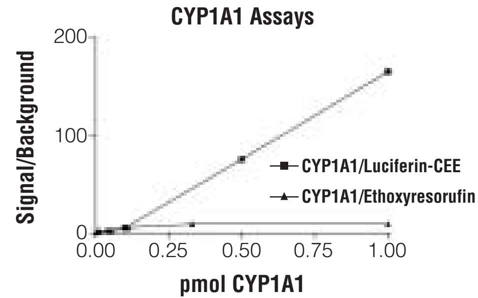
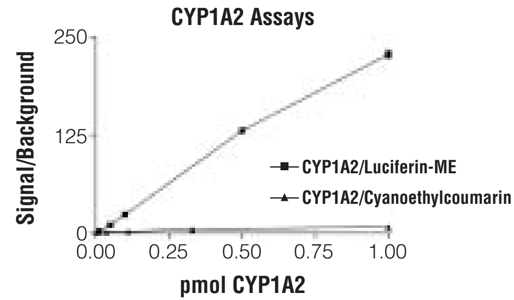
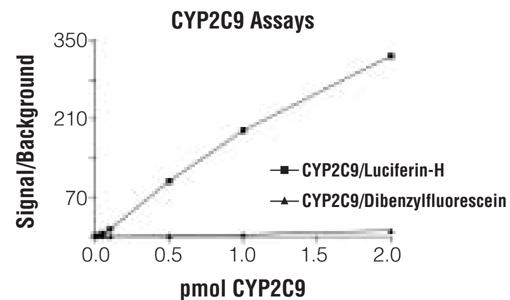


Figure 3. Using P450-Glo™ Assays to measure CYP450 inhibition. Recombinant forms of CYP450 were co-expressed in insect cells with CYP450 reductase or CYP450 reductase plus cytochrome b5 (Supersomes™ from BD/Gentest, Woburn, MA). CYP450 reactions were performed at 37°C in opaque white, flat-bottom 96-well plates (Costar®) in 50µl volumes in KPO_4 buffer (pH 7.4). Reaction mixtures were dispensed in wells and initiated by addition of an NADPH regenerating solution (BD/Gentest, Woburn, MA). CYP450 activity was stopped, and luminescence was initiated by adding Luciferin Detection Reagent. Luminescence was read directly on a FLUOstar Optima plate reading luminometer (BMG, Inc. Durham, NC). Values are the mean ± S.D., n = 3. (For additional assay details see Technical Bulletin #TB325).







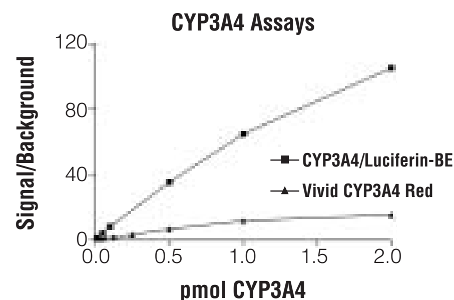


Figure 4. Comparison of P450-Glo™ luminescent assay to a fluorescent assay. CYP assays were performed with recombinant CYP450 membrane fractions (Supersomes™ from BD/Gentest, Woburn, MA) under optimized conditions at K_m for the fluorescent substrates and at or below K_m for the luminogenic substrates. For background measurements membrane fractions with no CYP450 activity were substituted for CYP450-containing membranes. CYP450 incubations with substrates were carried out 10–30 minutes at 37°C. Values represent the mean ± S.D., n = 3.

IN VITRO TOXICOLOGY

Soluble Substrates: Unlike many other CYP450 probe substrates, P450-Glo™ substrates are highly soluble in aqueous solutions.

Single Readout: By using a single luminescent readout, multiple CYP450 isoforms with multiple substrates can be assayed at a single instrument (luminometer) setting.

Low False-Positive Rate: Use of a proprietary stabilized firefly luciferase and luciferase assay formulation minimizes the incidence of false positives due to inhibition of luciferase by analytes when screening for CYP450 inhibitors.

P450-Glo™ Assays are available for several key human CYP450 isoforms including CYP1A1, 1A2, 2C8, 2C9, 3A4, 3A7 and 1B1. The P450-Glo™ CYP1A1 and 1A2 assays can also be used with rat CYP1A1 and 1A2, the CYP2C9 assay with rat CYP2C6 and 2C11, and the CYP3A4 assay with rat CYP3A1. The reactions work well with recombinant CYP450s (Figure 3) and with CYP450s in liver microsomes (data not shown).

An important use of CYP450 assays is detecting CYP450 inhibitors. P450-Glo™ Assays detect dose-dependent inhibition of recombinant CYP450s by known CYP450 inhibitors. Figure 3 provides examples in which P450-Glo™ luminogenic substrates were used as probes to detect inhibition of CYP1A1, 1A2, 2C9 and 3A4 by their respective inhibitors phenacetin, β-naphthoflavone, diclofenac and ketoconazole. IC_{50} values determined using P450-Glo™ Assays are similar to values obtained with conventional probe substrates (3). Single concentrations of multiple test compounds can be used when screening a chemical library, or a range of concentrations of a given compound can be screened to measure IC_{50} values.

The dynamic range and sensitivity of CYP450-Glo™ Assays are typically greater than fluorescent counterparts. Figure 4 provides comparisons between P450-Glo™ Assays and fluorescent alternatives. P450-Glo™ Assays gave superior signal:background ratios as well as improved sensitivity over the fluorescent assays.

P450-Glo™ CYP450 Assay Systems include a luminogenic CYP450 substrate that is supplied in an aqueous buffer, a lyophilized luciferin detection reagent and its reconstitution buffer. The user supplies a CYP450 preparation and its requisite co-factors. Kits are available in 10ml and 50ml sizes. Each 10ml system contains sufficient reagents for 200 assays at 50µl per assay in 96-well plates or 400 assays at 25µl per assay in 384-well plates. Each 50ml system contains sufficient reagents for 1,000 assays at 50µl per assay in 96-well plates or 2,000 assays at 25µl per assay in 384-well plates.

Conclusions

P450-Glo™ Assays provide a rapid, sensitive and highly reproducible approach to CYP450 screening. The systems are compatible with conventional CYP450 assay protocols in automated or manual multiwell plate formats and can be easily adapted for single-tube applications. P450-Glo™ Assays overcome many of the limitations of fluorescent and nonoptical methods by bringing the general advantages of luminescence technology to the study of CYP450s. ■

References

1. Guengerich, F.P. (2001) *Chem. Res. Toxicol.* **14**, 611–50.
2. Wienkers, L.C. and Hutzler, J.M. (2002) *Curr. Drug Disc.* 23–6.
3. Sai, Y. *et al.* (2000) *Xenobiotica* **30**, 327–43.

Protocol

P450-Glo™ CYP450 Assays Technical Bulletin #TB325 (www.promega.com/tbs/tb325/tb325.html)

Web site

www.promega.com/p450glo

[a]Patent Pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

P450-Glo is a trademark of Promega Corporation.

Costar is a registered trademark of Corning, Inc. Supersomes is a trademark of Bectin Dickinson/Gentest.

Ordering Information

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay[a,b]	10ml	V8751
	50ml	V8752
P450-Glo™ CYP1B1 Assay[a,b]	10ml	V8761
	50ml	V8762
P450-Glo™ CYP1A2 Assay[a,b]	10ml	V8771
	50ml	V8772
P450-Glo™ CYP2C8 Assay[a,b]	10ml	V8781
	50ml	V8782
P450-Glo™ CYP2C9 Assay[a,b]	10ml	V8791
	50ml	V8792
P450-Glo™ CYP3A4 Assay[a,b]	10ml	V8801
	50ml	V8802
P450-Glo™ CYP3A7 Assay[a,b]	10ml	V8811
	50ml	V8812