MINIATURIZING LUMINESCENT P450-GLO™ ASSAYS FOR HTS

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Determining toxicological properties of drug candidates is an important aspect of the drug discovery process. Using high-precision liquid handling instrumentation and robust, scalable reagent chemistries, toxicology assays can be brought closer to the high-throughput screening stages of drug development. We demonstrate the scalability of in vitro luminescent P450-Glo™ Assays for high-throughput cytochrome P450 activity screening in low-volume 384- and 1536-well formats.

Introduction

Scalable cell-based and biochemical assays are desirable for increasing throughput and decreasing assay costs in the drug discovery process. Designing such assays can be a challenge, requiring high-precision reagent dispensing, sensitive reading instrumentation and chemistries that can withstand scale-down to the desired assay volume.

We have demonstrated the successful miniaturization of the Promega P450-Glo™ Assays[a,b]. These assays can be used to assess drug effects on cytochrome P450 enzymes, key enzymes that are involved in the metabolism of therapeutic compounds. Incorporating P450 screening early in the drug discovery process expedites the detection of compounds that have undesirable toxicological characteristics before they are carried further into drug development.

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The "add-mix-measure" assay format of the P450-Glo™ Assays makes them ideal for miniaturization and automation. The assay procedure involves incubating cytochrome P450 enzymes with the desired test compound followed by incubation with luminogenic substrate. If the test compound has no or little inhibitory effect, the cytochrome P450 enzyme is able to generate luciferin. The luciferin in turn is metabolized by luciferase in a second reaction, ultimately generating light that is directly proportional to the activity of the P450 enzyme (Figures 1 and 2). In addition, signal half-life of over 2 hours allows batch processing of plates.

We were able to miniaturize the P450-Glo™ Assays using the Deerac Fluidics Equator™ nanoliter dispenser for non-contact delivery of sample and reagent. The multifunctional, PMT-based BMG LABTECH PHERAstar reader was used to detect assay signal. Select cytochrome P450 assays were performed in 3µl and 6µl volumes in 1536- and low volume 384 (LV384)-well formats, respectively. Z´-factor and known inhibitor screening data demonstrate robustness and functionality of the P450-Glo™ Assay.

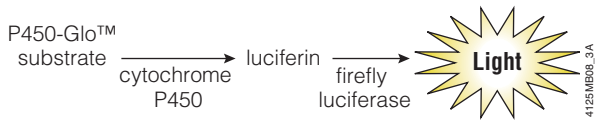

Figure 1. The P450-Glo™ Assay reaction scheme. CYP450 enzymes convert the P450-Glo™ luminogenic substrates to luciferin, a substrate for firefly luciferase. Luciferase catalyzes a reaction involving luciferase to produce light.

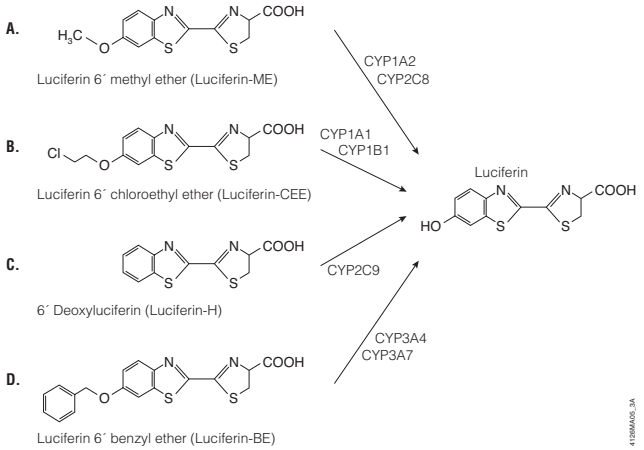

Figure 2. P450-Glo™ luminogenic substrates are converted to luciferin by CYP450 enzymes. Reaction A. The substrate for CYP1A2 and CYP2C8 is Luciferin-ME. **Reaction B.** The substrate for CYP1A1 and CYP1B1 is Luciferin-CEE. **Reaction C.** The substrate for CYP2C9 is Luciferin-H. **Reaction D.** The substrate for CYP3A4 and CYP3A7 is Luciferin-BE.

Methods

We used CYP1A1, CYP1A2, CYP2C9, and CYP3A4 recombinant cytochrome P450 preparations and NADPH regenerating system components A and B from BD Gentest. We followed the instructions for preparing the reaction mixtures provided in the *P450-Glo™ Assay Technical Bulletin* #TB325 (1). Before performing the assays and generating data presented here, we titrated each CYP450 enzyme to determine the minimal amount we could use for all reactions in the volumes to be tested (data not shown here). Table 1 lists the final reaction mixture components for each CYP450 reaction in 3µl and 6µl reaction volumes.

Table 1. Final Concentration of Reaction Components for P450-Glo™ Assays.

3µl Total Volume

CYP450 Isoform	Amount of CYP450	KPO_4 Concentration	Substrate Concentration
1A1	0.03125pmol	100mM	30µM Luciferin-CEE
1A2	0.03125pmol	100mM	100µM Luciferin-ME
2C9	0.0625pmol	25mM	100µM Luciferin-H
3A4	0.03125pmol	200mM	50µM Luciferin-BE

6µl Total Volume

CYP450 Isoform	Amount of CYP450	KPO_4 Concentration	Substrate Concentration
1A1	0.0625pmol	100mM	30µM Luciferin-CEE
1A2	0.0625pmol	100mM	100µM Luciferin-ME
2C9	0.125pmol	25mM	100µM Luciferin-H
3A4	0.0625pmol	200mM	50µM Luciferin-BE

The final reaction concentrations for the P450-Glo™ Assays were obtained from Technical Bulletin #TB325. The amount of CYP450 used for these miniaturized formats was determined by performing an enzyme titration (data not shown).

To perform the P450-Glo™ Assays, the reaction mixtures containing the cytochrome P450 enzymes, KPO_4 (except in the case of 3A4), and substrate were combined with either water or test compound (Table 1). Following a 10-minute incubation at room temperature, the NADPH-regenerating system (including KPO_4 for 3A4) was added to initiate the cytochrome P450 reaction and incubated for an additional hour at room temperature. Reconstituted Luciferin Detection Reagent (LDR) was then added to each well and incubated for 20 minutes. Relative Light Units (RLUs) were recorded with a plate reader (Table 2).

Z′-factor experiments were conducted to test the robustness of the assay and the pipetting accuracy of the Equator™ instrument when performing these assays in a miniaturized format (2). To accomplish this, each CYP450 enzyme was added to the designated wells. SF9 control membranes, also from BD Gentest, which lack the respective CYP450 enzyme, were added to an equal number of wells. Water was added to each reaction in the place of test compound. Differences in signal averages and standard deviations between the CYP450 reactions and SF9 control wells were used to compute the Z′-factor for each CYP450 isoenzyme.

A small drug screen using known cytochrome P450 inhibitors was also performed (3). Various concentrations of α-naphthoflavone, ketoconazole, or sulfaphenazole were added to the appropriate cytochrome P450 reaction mixture at a final concentration of 10µM. Vehicle-only reactions were used as assay controls for each CYP450/drug combination.

IC_{50} data were generated with each of the isoenzymes and their respective inhibitors. The test compounds were serially diluted manually and dispensed with the liquid handling instrument.

Table 2. Step-By-Step Volume Additions and Incubation Times for Minaturized P450-Glo™ Assays.

Step	For 3µl Volume	For 6µl Volume
1. Add Vehicle or test compound.	375nl	750nl
2. Add Cytochrome P450 reaction mixture.	375nl	750nl
3. Incubate 10 minutes.		
4. Add NADPH regenerating system.	750nl	1,500nl
5. Incubate 60 minutes.		
6. Add Luciferase Detection Reagent.	1,500nl	3,000nl
7. Incubate 20 minutes.		
8. Read Plates.		

The Deerac Fluidics Equator™ NS-808 Eight-Tip Pipetting system was used to dispense reaction components into low-volume plates designed for high-throughput applications. Three microliter total volume assays were performed in 1536-well plates (Corning Cat.# 3937). Six microliter total volume assays were prefomred in Low-Volume 384-well plates (Corning Cat.# 3673).

Automation for Low-Volume Dispensing

Miniaturization of screening assays requires fast, high-precision liquid dispensing. For this application we used the Deerac Fluidics Equator™ NS-808 Eight-Tip Pipetting System to dispense reaction mixtures and reagents for all of the assays. The Equator™ instrument is a fixed-tip, non-contact dispenser capable of accurate delivery of as little as 50nl of liquid. Using the Deerac Fluidics spot station software, methods were written that allowed dispensing in both nanoliter and microliter volumes into high-density plates using on-the-fly and stepped pipetting movements, respectively. Table 2 lists the volume additions at each step of the assays.

When automating assays, the viscosity of the liquids can play an important role in the success of the reagent delivery. Viscous liquids can clog dispensing tips and affect accuracy and precision of the assay. In the case of the P450-Glo™ Assays used here, all reagents used were amenable to automation down to nanoliter volumes, showing no tip clogging or bubble formation during delivery.

Detection

All plates were read using the BMG LABTECH PHERAstar plate reader. The PHERAstar is a multifunctional plate reader that uses photomultiplier tube (PMT) signal detection, even in high-density plate formats, such as 1536. A PMT-based reader is one option for labs that are moving toward higher throughput applications but do not need expensive CCD-based imagers.

Results and Discussion

Demonstrating HTS Assay Quality with Z´-Factor Analysis

Z´-factor data demonstrate assay variability and reproducibility in high-density plate formats. Z´-factor scores greater than 0.5 are reflective of excellent assays. The P450-Glo™ assays performed here show excellent Z´-factor values for all volumes tested (Table 3).

Screening for Inhibitors with P450-Glo™ Assay

The P450-Glo™ Assays can be used in screening applications for cytochrome P450 inhibitors. Using known cytochrome P450 inhibitory compounds as models, we are able to show that the P450-Glo™ Assays detect inhibition of the CYP enzymes (Table 4). This not only demonstrates proof-of-principle for each assay but also shows that the miniaturization of these assays does not compromise functionality. To determine percent inhibition, signal from wells containing test compound were compared to wells containing vehicle control.

Determining IC_{50} Values

Comparing the IC_{50} values between an assay and literature values using conventional substrates is another way to assess the functionality of an assay. IC_{50} data, the concentration of drug that shows 50% inhibition, was generated by serial dilution of a model inhibitor for each isoenzyme. GraphPad Prism® version 4.0 software was used for curve fitting and IC_{50} determination. The IC_{50} values obtained with the miniaturized P450-Glo™ Assays were comparable to data found in the literature (Table 5, Figure 3).

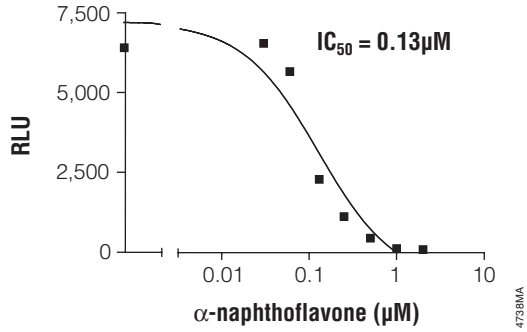

Figure 3. IC_{50} determination: Inhibition of CYP1A1 by α-naphthoflavone in 1536-well format. IC_{50} values were determined by serial dilution of the test compound with each CYP450 isoform. GraphPad Prism® was used for curve fitting and determining actual IC_{50} values for each assay.

Conclusions

We successfully automated and miniaturized P450-Glo™ Assays, generating data that shows the possibilities for using these assays in high-throughput drug screening applications. Most importantly, miniaturization of the P450-Glo™ Assays did not compromise assay quality, demonstrated by excellent Z´-factor scores and functional data using known cytochrome P450 inhibitors. ■

Table 3. Z´-Factor Values for Miniaturized P450-Glo™ Assays.

Cytochrome P450 Isoform	1536-Well Plate (3µl volume)	LV384-Well Plate (6µl volume)
1A1	0.83	0.81
1A2	0.72	0.74
2C9	0.64	0.71
3A4	0.71	0.76

Z´-factor values are all greater than 0.5, indicating excellent assays in both volumes tested (2).

Table 4. Inhibitor Screen Data for Miniaturized P450-Glo™ Assays.

CYP450 Isoform	Assay Format	Test Compound	Average RLU (Compound)	Average RLU (Vehicle)	% Inhibition
1A1	LV384	αNA	175	8,918	98
	1536	αNA	184	14,589	99
1A2	LV384	αNA	171	9,001	98
	1536	αNA	104	12,242	99
2C9	LV384	Sul	106	3,116	97
	1536	Sul	67	3,911	98
3A4	LV384	Ket	174	3,231	95
	1536	Ket	196	3,642	95

Model test compounds were screened against each CYP450 isoenzyme at a final concentration of 10µM. Percent inhibition was calculated for each isoform/test compound combination by dividing the average RLU value of wells containing the test compound by the average RLU value of wells containing the vehicle control and subtracting that value from 100. αNA = alpha-naphthoflavone, Sul = sulfaphenazole, Ket = ketoconazole.

Table 5. IC_{50} Values Obtained with Miniaturized P450-Glo™ Assays.

Cytochrome P450 Isoform/Substrate	IC_{50} (µM) αNA	Ket	Sul
CYP1A1/Luciferin-CEE (LV384)	0.11	–	–
CYP1A1/Luciferin-CEE (1536)	0.13	–	–
CYP1A1/ethoxycoum[3]	0.6	–	–
CYP1A2/Luciferin-ME (LV384)	0.13	–	–
CYP1A2/Luiciferin-ME (1536)	0.16	–	–
CYP1A2/imiaprine[3]	0.05	–	–
CYP2C9/Luciferin-H (LV384)	–	–	0.13
CYP2C9/Luicferin-H (1536)	–	–	0.13
CYP2C9/diazepam[3]	–	–	0.5
CYP3A4/Luciferin-BE (LV384)	–	0.09	–
CYP3A4/Luciferin-BE (1536)	–	0.05	–
CYP3A4/testosterone3	–	0.04	–

αNA = alpha-naphthoflavvone, Ket = ketoconazole, Sul = sulfaphenzole

[3]Sai, Y. *et al*. *Xenobiotica* 30, 327–43.

References

1. *P450-Glo™ CYP450 Assays Technical Bulletin* #TB325, Promega Corporation
2. Zhang, J. *et al.* (1999) *J. Biomol. Screen.* **4**, 67–73.
3. Sai, Y. *et al.* (2000) *Xenobiotica.* **30**, 327–43.
4. Cali, J. (2003) *Cell Notes* **7**, 2–4.

Protocol

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

Web Sites

www.promega.com/p450glo

www.deerac.com

www.bmglabtech.com

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

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents 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.