

Automated 384-Well Cell-Based Cytochrome P450 Inhibition Assays using Cryopreserved Human Hepatocytes in Suspension

Brad Larson¹, Peter Banks¹, Timothy Moeller², Tracy Worzella³, Mary Sobol³, Dongping Ma³, James J. Cali³

¹BioTek Instruments, Inc., Winooski, Vermont, USA • ²Celsis In Vitro Technologies, Baltimore, Maryland, USA • ³Promega Corporation, Madison, Wisconsin, USA

Overview

The ability to determine the enzymes and processes involved in drug metabolism, as well as the potential modulation of that metabolism, is essential to ensure proper therapeutic outcomes and eliminate potential drug-drug interactions (DDI). Though traditionally performed with microsomes, more recently hepatocytes have been utilized in order to provide more *in vivo*-like correlations. This desire, coupled with the fact that ADME/Tox assays in general are being run earlier in the drug discovery process, has led to the development of easy-to-use, robust assay chemistries, as well as the instrumentation to perform these assays in a high-throughput format.

Here we demonstrate an automated solution to run luminescent CYP450 inhibition assays using primary hepatocytes in a profiling format for CYP1A2, -2C9, and -3A4. Validation and pharmacology data prove how the combination of cells, assay, and instrumentation provide rapid, dependable information on the inhibition of CYP450-based drug metabolism in a cell-based format.

Introduction

DDI are of serious concern to the pharmaceutical industry and associated regulatory agencies. Cytochrome P450 (CYP) enzymes are key players in the metabolism of drugs within the body, and modulations in their activity have been implicated in many known DDI. Therefore, it is essential to understand how these enzymes can be affected by xenobiotics with regards to inhibition to avoid potential drug-drug interactions. Numerous formats currently exist to monitor the potential effects that a lead compound may have on a CYP. These include the use of recombinant CYP isoforms, microsomes, and immortalized cell lines with hepatocyte-like function. While each of these formats has its advantages, there is an increasing realization that data generated using these methods do not provide a complete picture of the effects a compound would have in an *in vivo* setting¹. Therefore, assays using primary hepatocytes are becoming increasingly important to accurately determine potential DDI. This is due to the fact that hepatocytes, the primary cell of the liver, possess the full complement of enzymes, nuclear factors and co-factors at physiological levels. Because of the increased desire to perform hepatocyte-based assays, coupled with the need to perform ADME-Tox assays earlier in the drug discovery process, it is essential to be able to perform these assays in a higher-throughput setting, using appropriate laboratory automation.

Here we present an application that demonstrates the ability to monitor CYP inhibition using cryopreserved human hepatocytes in an automated format. Using this setup, the inhibitory effects of lead compounds on CYP isoforms 1A2, 2C9, and 3A4 can be tested in a single 384-well plate, in a profiling format. Multiple luminescent assays, each using a luminogenic substrate specific for the isoform being analyzed, were employed for this application. The assay procedure was automated using a microplate dispenser as well as an 8-channel liquid handler. Four known inhibitors were tested with each of the three isoforms included in the profile, CYP1A2, -2C9, and -3A4. IC₅₀ values were derived from a similar 11-point titration curve. Inhibition data was compared to that generated using human liver microsomes in a similar assay format, as well as to IC₅₀ and K_m values from the literature, and the current FDA Draft Guidance.

BioTek Instrumentation

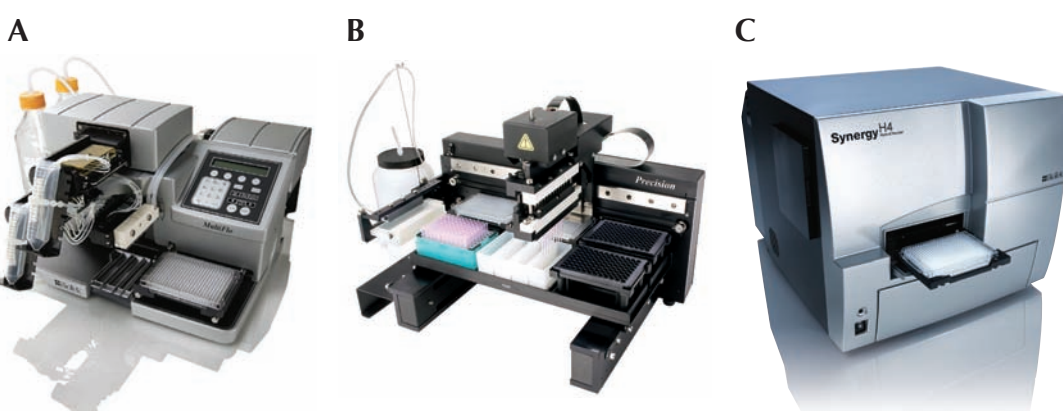


Figure 2 – CYP450 Inhibition Profiling Instrumentation.

A. MultiFlo™ Microplate Dispenser – The MultiFlo offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used to dispense cells, as well as to dispense chemistry components to the 384-well assay plates.

B. Precision™ Microplate Pipetting System – The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate across a 96-well PP plate, dilute the compounds to the correct 4X concentration, and then transfer 5 µL aliquots in quadruplicate from the 96-well compound plates to the 384-well assay plates.

C. Synergy™ H4 Hybrid Multi-Mode Microplate Reader – The Synergy H4 combines a filter-based and monochromator-based detection system in the same unit. The reader is capable of detecting a wide range of fluorescence signals, as well as high-performance luminescence detection. The instrument was used to read the luminescent signal from the P450-Glo™ assays.

P450-Glo Assay



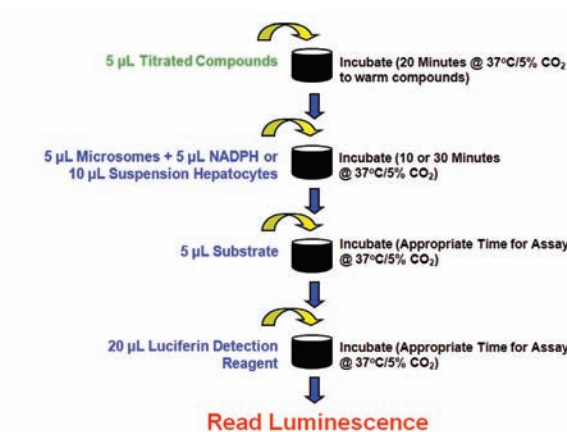
Figure 2 – The biochemical P450-Glo assay is performed by incubating titrated compounds with a luminogenic cytochrome P450 substrate, cytochrome P450 enzyme, and NADPH Regeneration System. The cell-based P450-Glo assay is similarly performed by incubating titrated compounds with luminogenic P450 substrate and hepatocytes. The substrates for this study are Luciferin-IPA for CYP3A4, Luciferin-H for CYP2C9 and Luciferin-1A2 for CYP1A2. The P450-Glo Substrates do not react with luciferase but are converted by cytochrome P450 to luciferin. Upon completion of the P450 reaction, detection reagent is added containing luciferase and ATP. These react with the luciferin created to produce light. The amount of light detected in the well is directly proportional to the amount of cytochrome P450 activity.

Suspension Hepatocytes

Hepatocytes are the most abundant cells of the liver and are involved in many critical functions of the body, including the majority of metabolism of endogenous and exogenous substances. Though cells lines derived from the liver, like HepG2 and HepaRG, are available, they lack the full complement of enzymes and transporters at physiologically relevant expression levels. Hepatocytes *in vitro* retain most of their *in vivo* function, especially phase I and phase II metabolism and transport activities and at physiologically relevant levels. Due to these attributes, hepatocytes are recognized as the gold standard for determining drug metabolism and safety profiling by researchers, pharmaceutical industry and regulatory agencies.

Hepatocytes may be used from fresh isolations or from cryopreserved preparations. Hepatocytes are utilized in suspension cultures for studies lasting for less than six hours such as metabolic stability, inhibition of metabolic enzymes or uptake transporter assays. Though animal hepatocytes are employed to study ADME properties of new chemical entities, species-specific difference make correlation to human activities difficult. The use of cryopreserved human hepatocytes provide an ideal reagent to study human-specific metabolic profiling and DDI. Cryopreserved human hepatocytes, and not freshly isolated preparations, allow for scheduled experiments and use the same donor in repeated studies, which are critical for screening assays in drug discovery.

Optimized Automated Assay Procedure



Blue Text: MultiFlo Dispensing
Green Text: Precision Titration and Transfer
Red Text: Synergy H4 Microplate Reads

Figure 3 – P450-Glo Automated Assay Protocol.

Automated Assay Validation

Microsome/Suspension Hepatocyte Dispensing

The ability of the MultiFlo to accurately dispense human liver microsomes and suspension hepatocytes to 384-well plates was verified by analyzing the %CV of values across all reaction wells in the assay (384-well: Columns 1-22). For the microsome dispense test, a CYP3A4 P450-Glo assay was run using a final microsome concentration of 0.02 µg/µL. For the hepatocyte dispense test, 10 µL of cells at a concentration of 1x10⁶ cells/mL were dispensed into the plate, followed by 10 µL of CellTiter-Glo® reagent. The reagent lyses the cells and measures cell number based on ATP content in the well. %CV values were 7.55% for microsome dispensing, and 3.58% for hepatocyte dispensing.

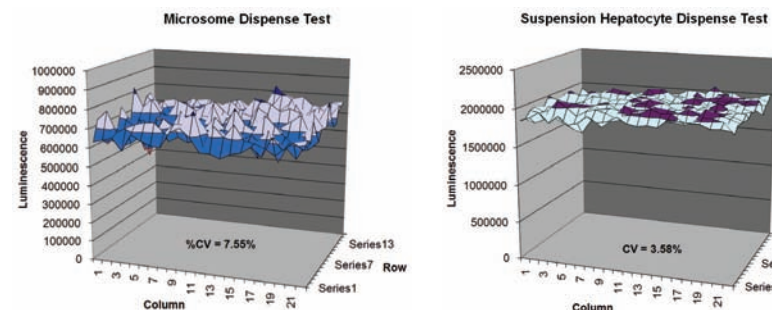


Figure 4 – Plating efficiency of microsomes and suspension hepatocytes by MultiFlo.

Automated Assay Validation

Z'-Factor Validation

A Z'-Factor assay was performed to validate the automated CYP2C9, -3A4, and -1A2 assay procedures using human liver microsomes and suspension hepatocytes. Known inhibitors (Sulfaphenazole:CYP2C9; Ketoconazole:CYP3A4; Alpha Naphthoflavone:CYP1A2) were used at 0 µM or 10 µM concentrations for the positive and negative controls, respectively.

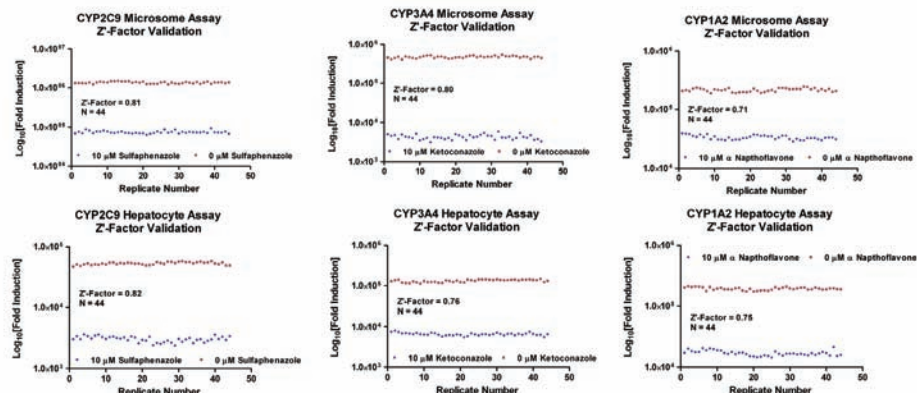


Figure 5 – Z'-Factor validation data. A Z' value ≥ 0.5 is indicative of an excellent assay².

CYP Compound Profiling Setup

Twelve inhibitors were profiled against three separate CYP enzymes. Four known inhibitors were selected for each of the three CYPs included in the profile (Sulfaphenazole, Fluconazole, Diclofenac, Fluconazole:CYP2C9; Ketoconazole, Verapamil, Troleandomycin, Ritonavir:CYP3A4; Alpha Naphthoflavone, Furafylline, Fluvoxamine, Propranolol:CYP1A2). Eleven point dose responses were generated for each compound, including a no compound control. Each individual compound titration was run with the three P450-Glo assays, using either human liver microsomes or suspension hepatocytes and the automated process previously described. The entire process was repeated a total of three times each with microsomes and hepatocytes, on separate days with fresh aliquots of compound.

Microsome-Based Assay Compound Profiling

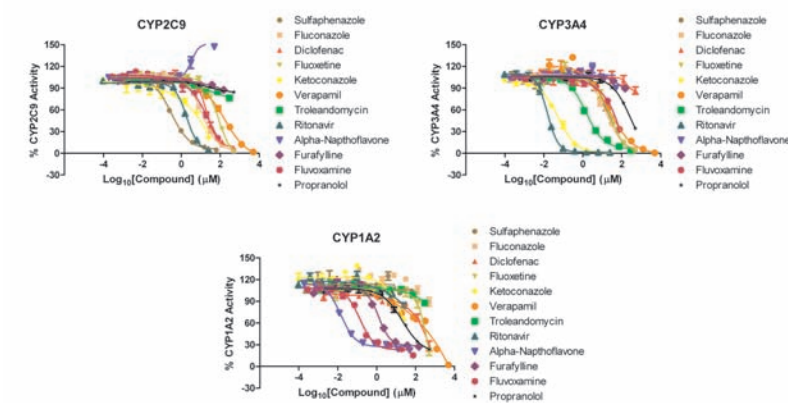


Figure 6 – Representative compound inhibition curves generated with the CYP2C9, -3A4, and -1A2 microsome-based assays.

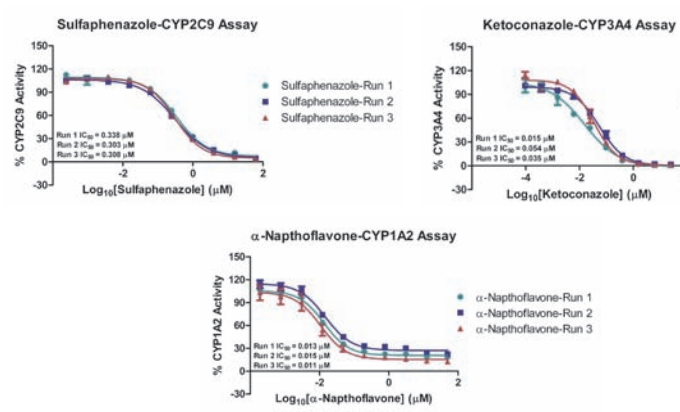


Figure 7 – Multi-run individual compound inhibition curves.

	Target Assay	Literature IC ₅₀ Value (μM)	Automated Microsome-Based P450-Glo Assays		
			2C9	3A4	1A2
Diclofenac	2C9	3.4-52 (K _m) ³	18.96 ± 4.04		
Fluconazole	2C9	7 ³	10.96 ± 1.74		
Fluoxetine	2C9	18-41 ³	73.80 ± 9.42		
Sulfaphenazole	2C9	0.3 ³	0.32 ± 0.019		
Ketoconazole	3A4	0.0037-0.18 ³		0.035 ± 0.019	
Ritonavir	3A4	0.041-0.9 ³		0.016 ± 0.001	
Troleandomycin	3A4	0.5 ⁴		1.26 ± 0.26	
Verapamil	3A4	10-24 ³		17.45 ± 4.11	
Alpha-Naphthoflavone	1A2	0.01 ³			0.013 ± 0.002
Fluvoxamine	1A2	0.24-0.48 ⁵			0.114 ± 0.032
Furafylline	1A2	0.6-0.73 ¹			1.20 ± 0.097
Propranolol	1A2	8.9-77.5 (K _m) ⁶			16.52 ± 5.11

The data shows the ability of the automated process to provide inhibition data for numerous compounds with multiple CYP assays (Figure 6) in a single run using human liver microsomes. It can also be seen (Figure 7) that the titration process and assays are repeatable as witnessed by the similarities in inhibition curves and IC₅₀ values across multiple runs. Finally, the data in Table 1 once again illustrates the repeatability of the automated profiling process, and also demonstrates the accuracy in the pharmacology data for each compound. IC₅₀ values are within ½ log of existing literature values, which have been taken from the 2006 FDA Draft Guidance for Drug Interaction Studies³, or other previously published values. Literature references are IC₅₀ values with the exception of Diclofenac and Propranolol, which are substrates for CYP2C9 and -1A2, respectively. The values listed here represent K_m values for the substrate, although one would expect these values to be similar when these compounds are used as competitive inhibitors, as they are for this project.

Hepatocyte-Based Assay Compound Profiling

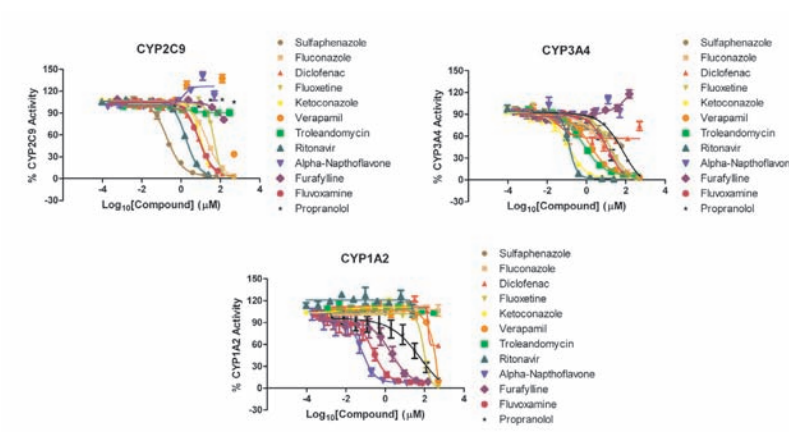


Figure 8 – Representative compound inhibition curves generated with the CYP2C9, -3A4, and -1A2 hepatocyte-based assays.

Target Assay	Literature IC ₅₀ Value (μM)	Automated Microsome-Based P450-Glo Assays			
		2C9	3A4	1A2	
Diclofenac	2C9	3.4-52 (K _m) ³	18.96 ± 4.04		
Fluconazole	2C9	7 ³	10.96 ± 1.74		
Fluoxetine	2C9	18-41 ³	73.80 ± 9.42		
Sulfaphenazole	2C9	0.3 ³	0.32 ± 0.019		
Ketoconazole	3A4	0.0037-0.18 ³		0.035 ± 0.019	
Ritonavir	3A4	0.041-0.9 ³		0.016 ± 0.001	
Troleandomycin	3A4	0.5 ³		1.26 ± 0.26	
Verapamil	3A4	10-24 ³		17.45 ± 4.11	
Alpha-Naphthoflavone	1A2	0.01 ³			0.013 ± 0.002
Fluvoxamine	1A2	0.24-0.48 ⁵			0.114 ± 0.032
Furafylline	1A2	0.6-0.73 ¹			1.20 ± 0.097
Propranolol	1A2	8.9-77.5 (K _m) ⁶			16.52 ± 5.11

Table 2 – IC₅₀ values for known inhibitors of each hepatocyte-based assay. Results from three individual runs are shown for each compound. Values shown represent µM concentrations.

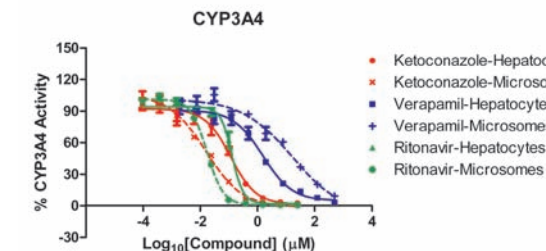


Figure 9 – Representative compound inhibition curves using suspension hepatocytes or human liver microsomes.

The results in Figure 8 demonstrate how the automated profiling process can be adapted to a cell-based assay format. Standard deviations from IC₅₀ or K_m values generated across multiple runs (Table 2) remain small, and agree with existing literature values. It can also be seen by examining the averages listed in Table 1 and 2, as well as the inhibition curves plotted in Figure 9, that significant differences do exist between data generated using a purified CYP enzyme setting, such as human liver microsomes, and that generated using human hepatocytes. For example, Verapamil IC₅₀ is lower in hepatocytes presumably due to transporter affect of increasing intracellular concentrations of the inhibitor compared to microsomal activity. This supports the notion that processes which take place within an intact, functioning cell, such as uptake and efflux drug transport, can affect the inhibitory properties of a compound, and illustrates the need to perform future CYP450 inhibition assessments in a cell-based format.

Conclusions

1. Suspension human liver hepatocytes provide an easy-to-use method to measure the inhibitory effects of compounds on Cytochrome P450 enzymes.
2. The P450-Glo assay allows for rapid and accurate assessment of the inhibitory effects of compounds with multiple CYP enzymes in a profiling format.
3. The instrumentation used enables the CYP profiling process to be automated in a way which improves throughput, yet still provides a robust assay process.
4. Comparative results from microsomes and hepatocytes demonstrate the inhibitory differences which can be seen between the two formats, and illustrate the need to perform CYP inhibition assessments in a cell-based manner.