

Introduction

The ability to determine the effects that lead compounds have on cytochrome P450 enzymes is an important part of today's drug discovery routine. One desired component of this process is the ability to profile compounds against multiple CYP450 enzymes using the same basic procedure. The second is an easy, yet dependable way to dilute compounds that will create accurate titration curves.

Here we demonstrate the automation of dose-response curves using BioTek's Precision™ XS Automated Pipetting System. IC₅₀'s of small molecule drugs were determined using recombinant CYP isoforms 3A4, 2C9 and 2D6 and luminogenic substrates specific to each from Promega's P450-Glo™ CYP450 Screening Systems. Compounds were profiled against all three isoforms on the same 384-well assay plate to demonstrate the ease of this combined procedure.

Abstract

Most small molecule drugs are metabolized predominantly in the liver by cytochrome P450 (CYP) enzymes, particularly CYP isoforms 3A4, 2C9 and 2D6. It is important to assess metabolism for appropriate dosing, but also for establishing metabolism-related drug-drug interactions where one drug may affect the metabolism of another, leading to possible toxic effects. While the gold standard method for *in-vitro* determination of lead compound inhibition of CYP isoforms involves monitoring the metabolism of drug substrates by human liver microsomes or primary hepatocytes using LC-MS/MS, there is a growing need and desire for a low cost, higher throughput, and easily automatable alternative that can assess metabolic profiles of leads earlier in the drug discovery process.

Promega's P450-Glo™ CYP450 Screening Systems, combined with BioTek's instrumentation, provides such a solution. The P450-Glo™ assays use recombinant human cytochrome P450 enzymes, and luminogenic cytochrome P450 substrates appropriate for the enzyme being tested, to assess the effects of compounds on cytochrome P450 activity. BioTek's liquid handling and detection instruments provide sensitive, flexible, and user friendly ways to automate compound titration and microplate formatting steps, as well as read the luminescent output from the assay. This combination of chemistry, robotics, and detection creates an ideal solution for high-throughput cytochrome P450 profiling of lead compounds in drug discovery campaigns.

P450-Glo™ Luminescent CYP450 Assay



Figure 1 – The P450-Glo™ assay was performed by incubating a luminogenic cytochrome P450 substrate with a cytochrome P450 enzyme and NADPH Regeneration System. The P450-Glo™ Substrates do not react with luciferase but are converted by cytochrome P450 to luciferin, which in turn reacts with luciferase to produce light. The amount of light detected in the well is directly proportional to the amount of cytochrome P450 activity.

BioTek Instrumentation

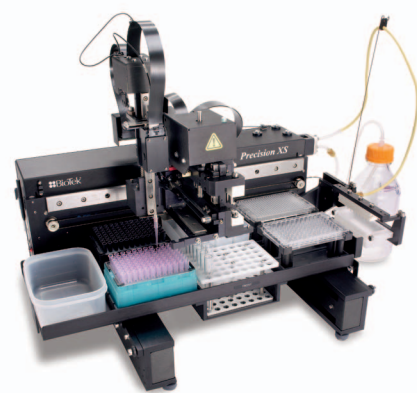


Figure 2 – Precision™ XS Microplate Sample Processor

The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate two compounds 1:2, in triplicate, across a 96-well microplate. Four 6 µL aliquots of each compound concentration were then transferred to the 384-well assay plate.



Figure 3 – Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader

The Synergy™ Mx Multi-Mode Reader's quadruple monochromator system employs Ultra Fine-Tuned™ technology to select wavelengths with a repeatability of ±0.2 nm. Its top optical head can focus up and down on the samples with a 100 µm resolution. Four slits on the excitation and emission side provide a choice of 16 bandpass combinations for every wavelength pair. The instrument includes dedicated luminescence optics with a low-noise PMT detector.

Precision™ XS Compound Titration Method

Automated Method

1. Transfer 60 µL of 1% DMSO from a 96-well deep well microplate to columns 2-12 of rows A-C and E-G of the 96-well microplate.
2. Aspirate 30 µL from column 1, containing the highest concentration of test compound, dispense to column 2, and perform a 10X mix.
3. Repeat the procedure for columns 2-11, leaving column 12 as a no compound control.
4. Transfer four 6 µL aliquots from each well of the 96-well microplate to the 384-well assay plate.

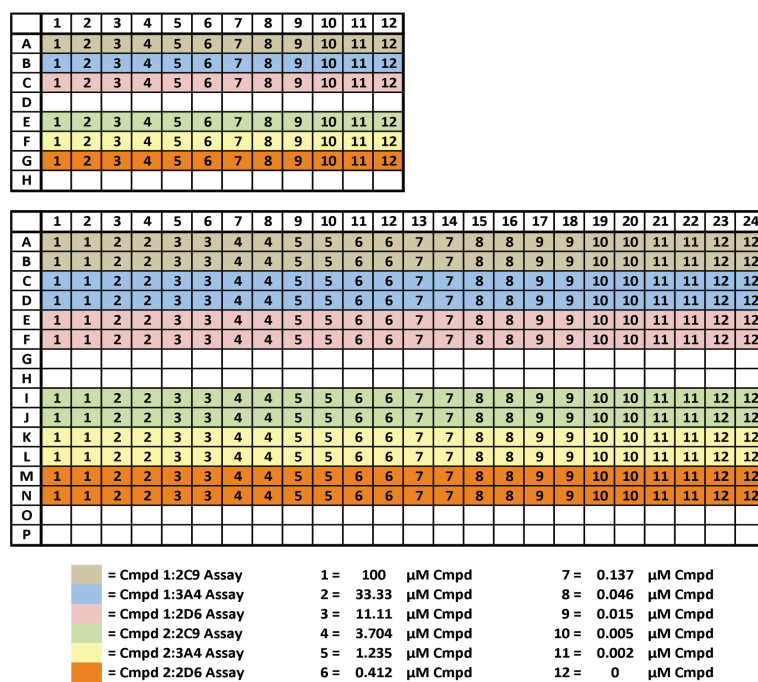


Figure 4 – Plate layout for Precision™ XS automated method.

The linearity of the titration method was tested using ATP in 1% DMSO, beginning with a top concentration of 100 µM. After the 6 µL transfers to the 384-well microplate, an equal volume of Promega's Kinase-Glo® Plus reagent was manually added to the wells. Luminescence was measured on the Synergy™ Mx after a 10 minute incubation.

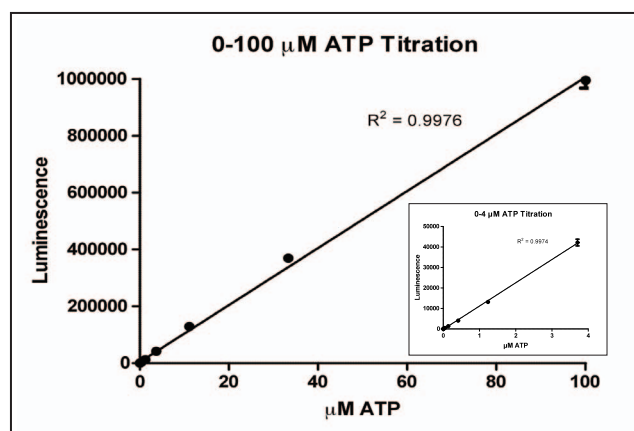


Figure 5 – Graphs showing linearity of Precision™ XS compound titration method across titration range of 0-100 µM ATP.

P450-Glo™ Assay Validation

Z'-Factor assays were performed in order to validate the CYP2C9, 2D6, and 3A4 P450-Glo™ Screening Systems in 384-well format prior to inhibitor testing. Component conditions and incubation times used are listed in Table 1. Microplate shaking was performed using the Synergy™ Mx, and incubations were done at room temperature in order to simulate potential lab conditions.

Experimental Setup				
Cytochrome P450	CYP per Reaction 384-Well Plate (Total pmol)	Potassium Phosphate Conc. (mM)	Substrate Concentration (K _m Conc.)	Room Temp. Incubation Time (Min.)
CYP2C9	0.25 pmol	25 mM	100 µM Luciferin-H	75
CYP2D6	0.125 pmol	100 mM	30 µM Luciferin-ME EGE	45
CYP3A4	0.05 pmol	100 mM	3 µM Luciferin-IPA	10

Table 1 – Component concentrations and incubation times for P450-Glo™ assays.

1. 6 µL of 1% DMSO manually dispensed to microplate.
2. 6 µL of CYP450, substrate, KPO4 solutions manually dispensed to microplate.
3. 12 µL of NADPH Regeneration System manually dispensed to plate. Microplates shaken for 60 seconds using linear shaker on Synergy™ Mx, and incubated at RT for appropriate times listed in Table 1.
4. 24 µL of Luciferin Detection Reagent manually added to microplate, followed by 60 second Synergy™ Mx linear shake, and 20 minute RT incubation.
5. Luminescence read on Synergy™ Mx.

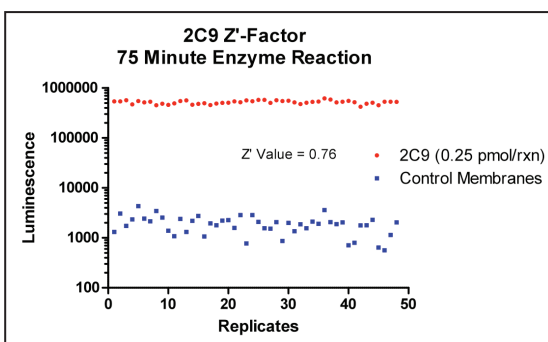


Figure 6 – Z'-Factor validation results for CYP2C9, 2D6, and 3A4 P450-Glo™ Assays.

CYP450 Inhibitor Profiling

Final testing of BioTek instrumentation involved profiling test compounds against the three CYP450 isoforms. Two compounds were tested on a single assay plate using the three validated P450-Glo™ assays.

Experimental Setup

1. Use the Precision™ XS to titrate test compounds, and transfer to the 384-well assay plate.
2. Run the P450-Glo™ assays against each test compound on the microplate.
3. Read luminescence on Synergy™ Mx and compute IC₅₀ values.

Results

Compound	P450-Glo IC ₅₀ Values (µM)			Control Assay IC ₅₀ Values (µM)		
	2C9	3A4	2D6	2C9	3A4	2D6
Sulfaphenazole	0.08	23.61	No Effect	0.18 ^[36]		
Diclofenac	1.572	No Effect	No Effect	2.8 ^[106]		
Ketoconazole	4.711	0.083	42.75		0.05 ^[41]	
Nifedipine	1.395	2.786	38.03		3.2 ^[77]	
Quinidine	No Effect	81.18	0.005			0.009 ^[38]
Dextromethorphan	No Effect	>100	5.293			7 ^[48]

Table 2 – IC₅₀ values for compounds tested with P450-Glo™ assays.

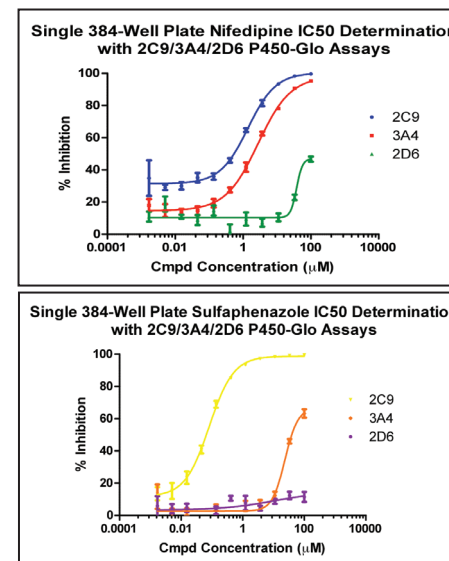


Figure 7 – CYP450 profiling results for two compounds run on a single 384-well assay plate.

Conclusions

1. The Precision™ XS provides an easy-to-use solution to deliver accurate compound titrations for IC₅₀ determination.
2. The sensitivity of the optical system in the Synergy™ Mx is able to dependably read luminescence output from a 48 µL reaction in 384-well format.
3. Promega's P450-Glo™ Screening Systems provide rapid, high-throughput CYP450 inhibition data that agrees to a high degree with established literature values.
4. The combination of BioTek's instrumentation, and Promega's P450-Glo™ Screening Systems create an ideal solution for high-density, automated cytochrome P450 profiling.