

Combining Luminescence-based CYP Inhibition Assays and Simple, Robust Instrumentation for Use in Automated Cytochrome P450 Profiling



Brad Larson¹, Peter Banks¹, Mary Sobol², James J Cali²

¹BioTek Instruments, Inc., Winooski, Vermont, USA • ²Promega Corporation, Madison, Wisconsin, USA



Promega

Introduction

The determination of effects that lead compounds have on cytochrome P450 (CYP) enzymes is an important part of ADME/Tox testing. Current methods involve running one, or multiple compounds, in dose response format against a single CYP enzyme. This process is time consuming, and can lead to data set variability due to assay and day-to-day laboratory variability.

The automated assay shown here eliminates these concerns and provides the ability to profile multiple compounds against the CYP2C9, -2D6, and -3A4 isoforms on the same 384-well assay plate using one simple process.

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 inhibit 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, the use of recombinant CYP isoforms with optical readouts based on labeled drug substrates specific to the isoform is gaining favor as a low cost, higher throughput alternative that can assess metabolic profiles of leads earlier in the drug discovery process. One desired component of this change is the ability to profile compounds against multiple CYP 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 the profiling process, from compound titration through assay component transfer, using simple, yet robust instrumentation. IC₅₀'s of small molecule drugs were determined using recombinant CYP isoforms 3A4, 2C9 and 2D6 as well as luminogenic substrates specific to each. Compounds were profiled against all three isoforms on the same 384-well assay plate to demonstrate the ease of this combined procedure. The combination of chemistry and instrumentation creates an ideal solution for high-throughput cytochrome P450 profiling of lead compounds in drug discovery campaigns.

BioTek Instrumentation

A. The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well polypropylene plate, as well as dispense all assay components to the 384-well assay plates.

B. The Synergy™ Mx Monochromator-based Multi Mode Microplate Reader incorporates a quadruple monochromator system along with a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. The instrument was used to detect the luminescent P450-Glo™ signal from each assay well.

P450-Glo™ Luminescent CYP450 Assay



Figure 1 – The P450-Glo assay was performed by incubating titrated compounds with a luminogenic cytochrome P450 substrate, 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.

Precision Compound Titration Method

A serial 12-point 1:4 titration method was set up on the Precision to be used for compound dilution.

The titration method's linearity was tested using ATP in 1% DMSO, beginning with a top concentration of 100 μM. 5 μL transfers were performed in quadruplicate for each point in the titration to the 384-well microplate. An equal volume of Promega's Kinase-Glo® Plus reagent was then manually added to the wells. Luminescence was measured on the Synergy Mx after a 10 minute incubation.

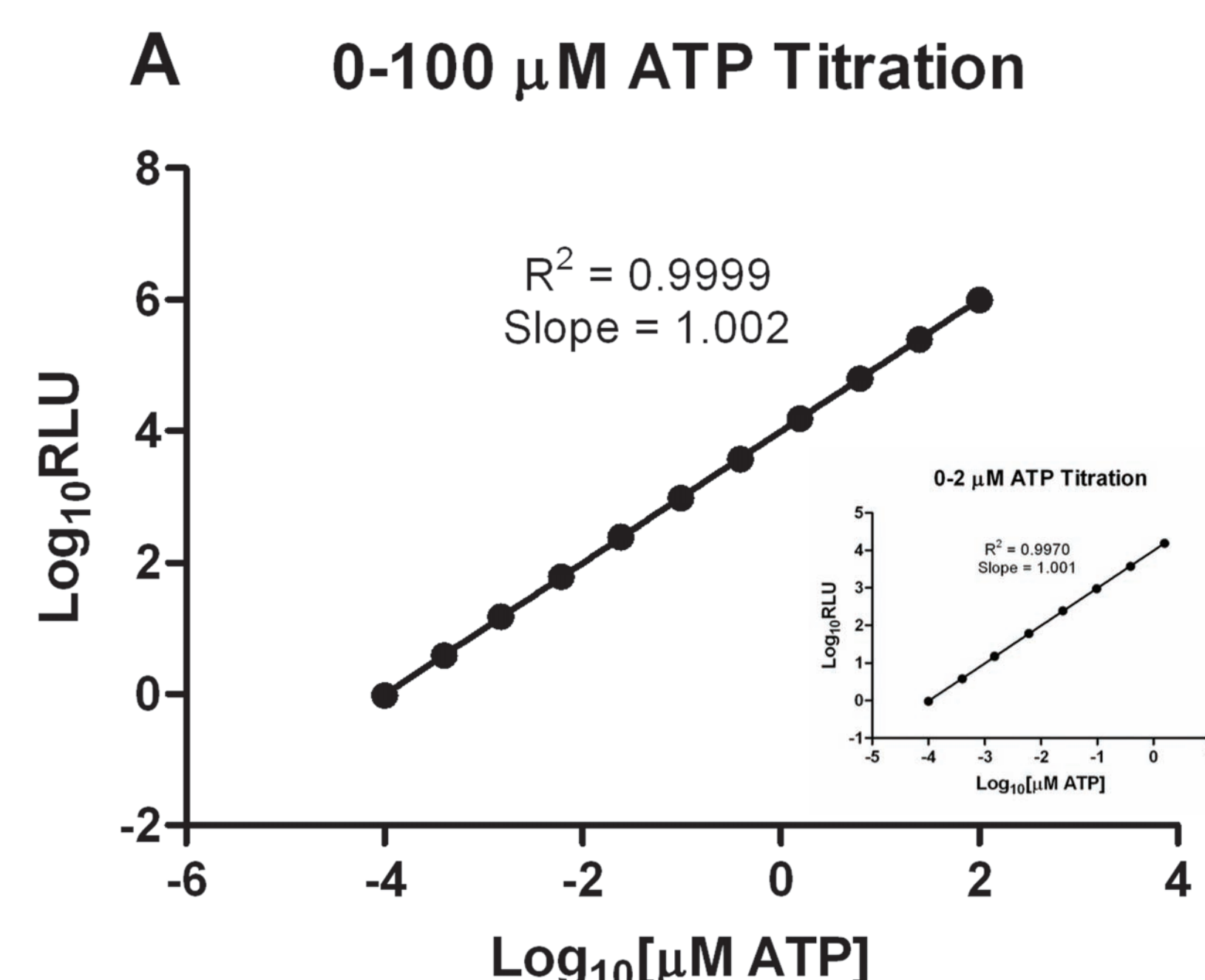


Figure 2 – Luminescent values from Precision 0-100 μM ATP titration.

R² and slope values indicate that a consistent 1:4 dilution is attained across the entire titration range.

P450-Glo Assay Automated Workflow

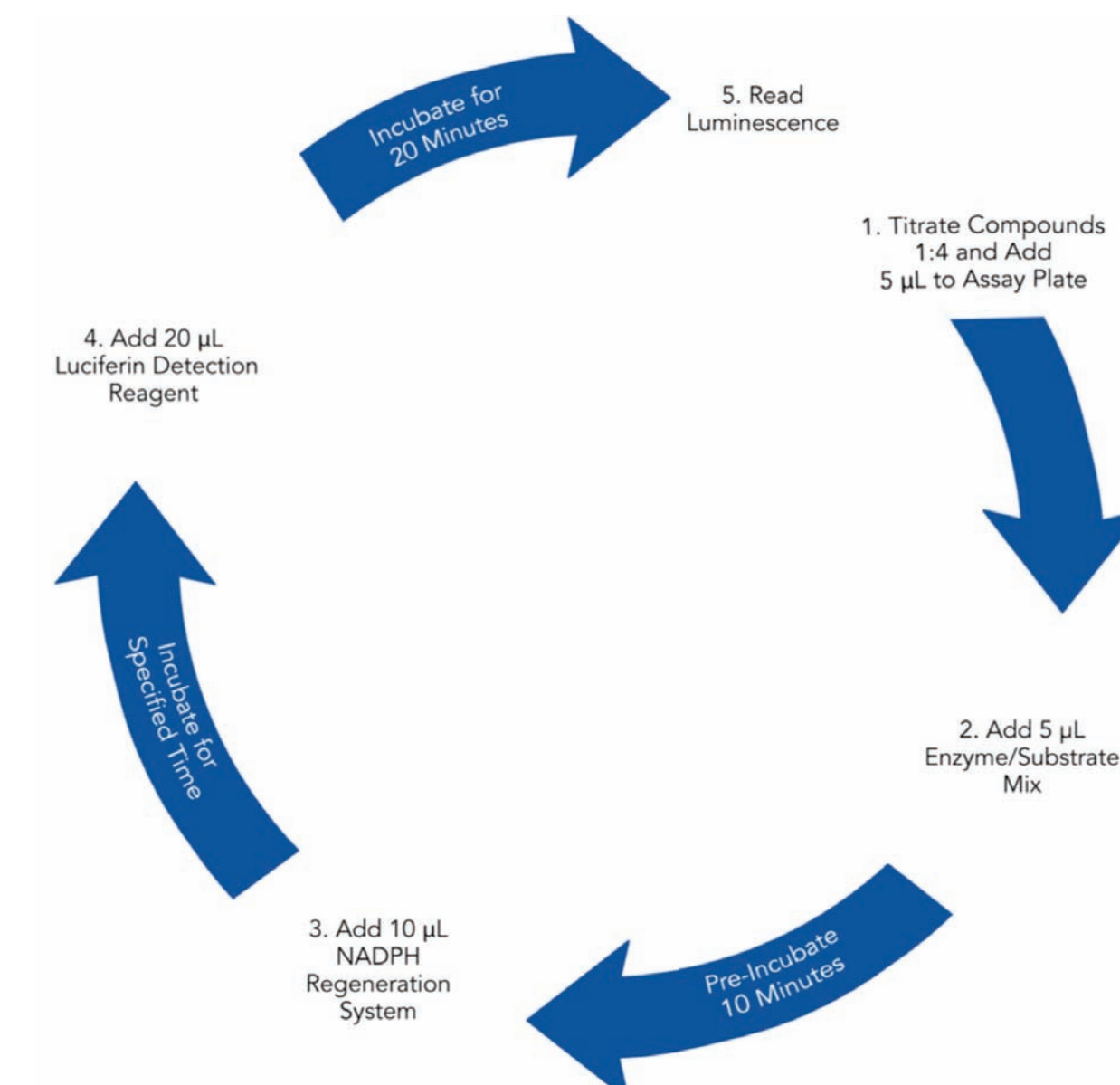


Figure 3 – All compound titrations and assay component dispensing were performed by the Precision. All plate reads were performed by the Synergy Mx. Plates were shaken for 30 seconds at 1000rpm following each component addition. Incubations were performed at room temperature.

P450-Glo Assay Validation

Z'-Factor assays were performed in order to validate the CYP2C9, 2D6, and 3A4 P450-Glo automated assays in 384-well format prior to inhibitor testing. Component conditions and incubation times used are listed in Table 1.

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.

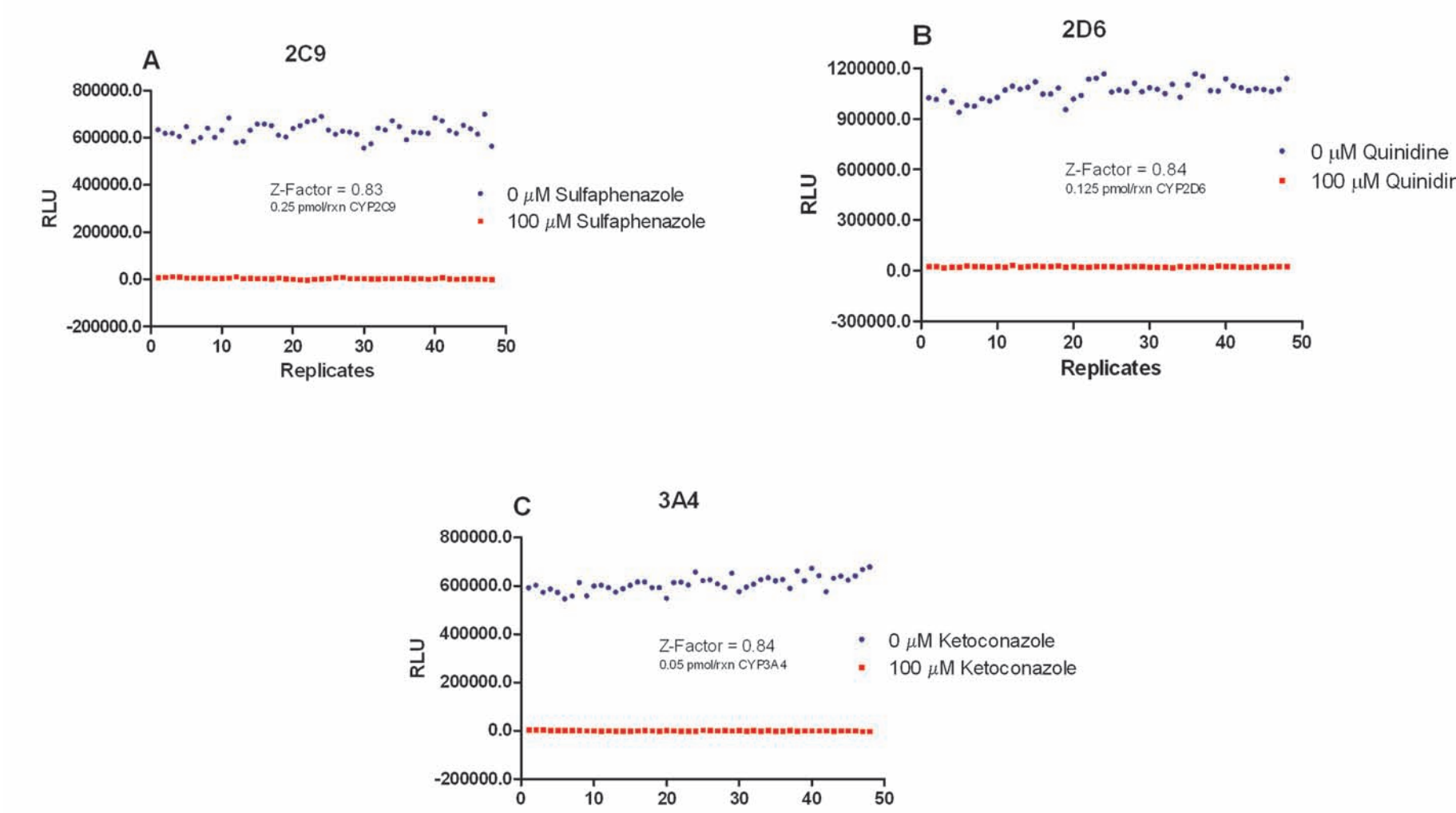


Figure 4 – Z'-Factor validation results for automated CYP2C9, 2D6, and 3A4 P450-Glo Assays.

CYP450 Inhibitor Profiling

Final testing of the automated P450-Glo assays 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.

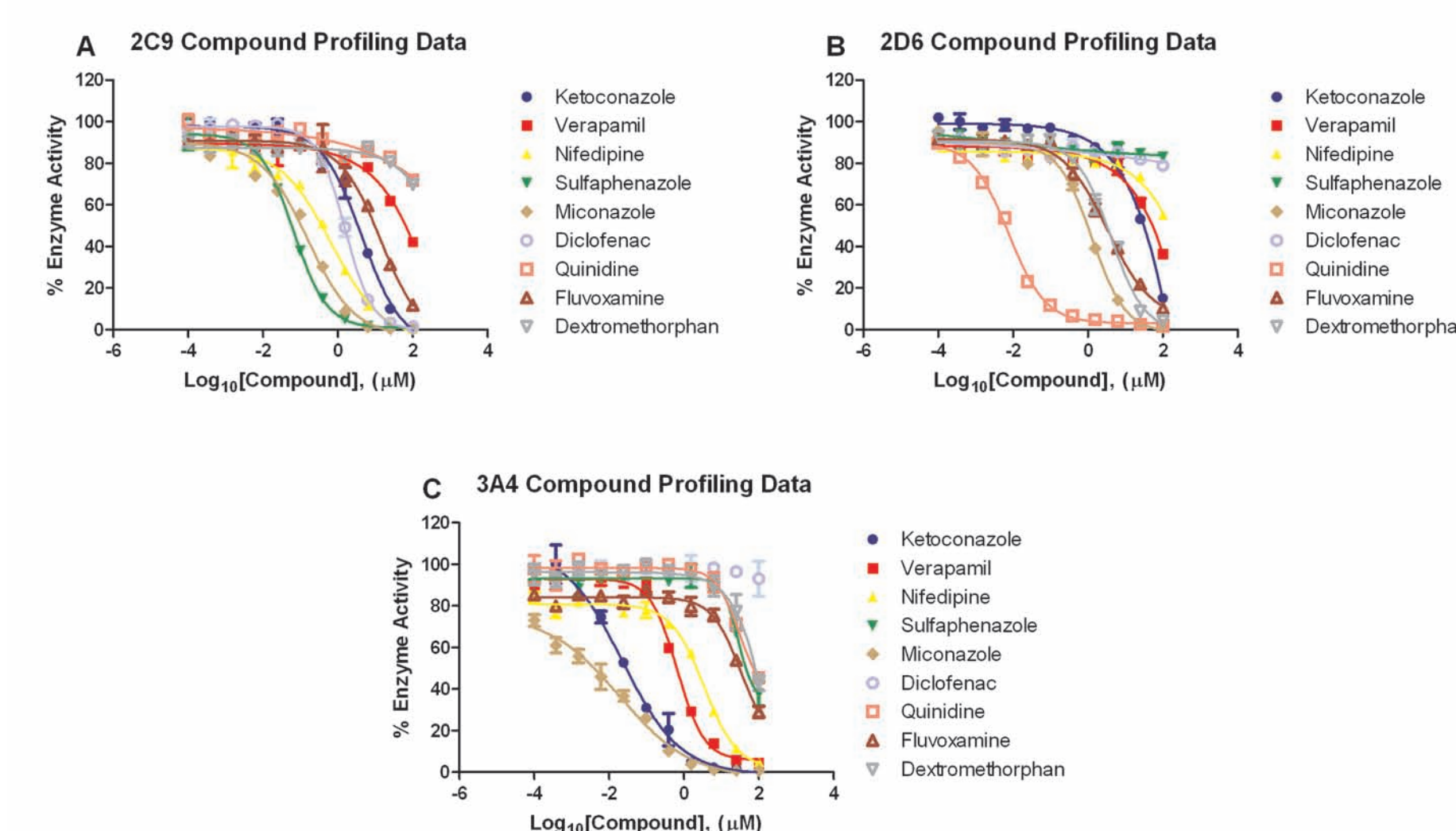


Figure 5 – Compound profiling inhibition curves for automated CYP2C9, 2D6, and 3A4 P450-Glo Assays.

Compound	P450-Glo IC ₅₀ Values (μM)			Control Assay IC ₅₀ Values (μM) ^a		
	2C9	2D6	3A4	2C9	2D6	3A4
Sulfaphenazole	0.07	No Effect	28.06	0.18 ^[38]		
Diclofenac	1.53	No Effect	No Effect	2.8 ^[108]		
Miconazole	0.16	1.28	0.02	0.5 ^[138]		
Ketoconazole	4.32	>100	0.02			0.05 ^[41]
Nifedipine	0.68	>100	3.46			3.2 ^[477]
Verapamil	>100	>100	0.66			1.8 ^[472]
Quinidine	No Effect	0.007	30.6		0.009 ^[38]	
Dextromethorphan	No Effect	4.09	>100		7 ^[48]	
Fluvoxamine	14.14	3.3	32.68		4.9 ^[87]	

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

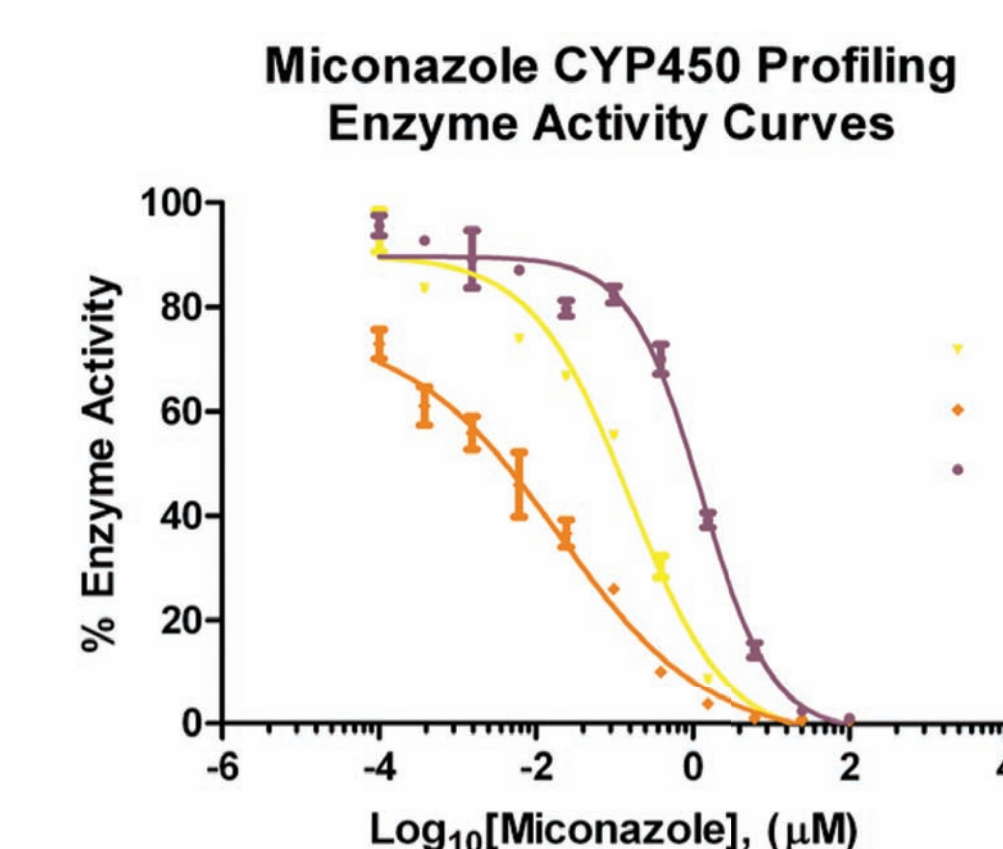


Figure 6 – Miconazole CYP450 profiling results.

As results are brought together, the effects a compound will have on the different CYP enzymes become apparent, or how an individual enzyme will be affected by each lead compound. An example is the antifungal drug miconazole. This compound is a well known inhibitor of CYP2C9^[39]. However, as Figure 6 shows, it is also a potent inhibitor of CYP3A4 and -2D6, which also agrees with the literature^{c,d}.

Conclusions

1. The Precision 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 40 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.