

## IN VITRO COMPOUND PROFILING IN 384- AND 1536-WELL FORMATS: USING BIOLUMINESCENT ADME ASSAYS TO UNDERSTAND IN VIVO BIOLOGY

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This article describes metabolic profiling applications in which compounds are assayed against several different drug metabolizing enzymes on the same assay plate. We obtain potency data for cytochrome P450 and monoamine oxidase A using this parallel approach and dose-response testing of compounds using both 384-well and 1536-well plate configurations.

### Introduction

Cytochrome P450 (CYP450) and monoamine oxidase (MAO) enzymes are important catalysts of oxidative drug metabolism (1). Oxidative metabolism increases the aqueous solubility of drugs, rendering them more amenable to elimination from the body.

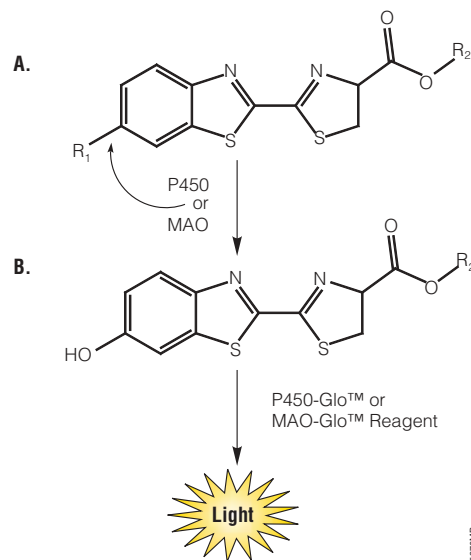
Drug-metabolizing enzymes play a central role in adverse drug-drug interactions (DDIs), which occur when the metabolism of one drug is increased or decreased by the administration of a second drug. When the rate of a drug's metabolism is decreased, its concentration in a patient can increase to a toxic level. Conversely, the therapeutic efficacy of one drug is reduced when its metabolism is accelerated by the administration of a second drug. Both types of DDI represent significant liabilities for any medicine.

### Luminescent Assays for Profiling

Screening compounds for their impact on metabolic activities is an important aspect of the drug discovery process. If this can be done early in the process, costly false starts due to metabolic liabilities can be avoided.

Bioluminescent assays for drug-metabolizing enzymes offer many advantages for compound profiling. Low luminescent assay background results in high signal-to-noise ratios, such that only small amounts of enzyme are used per reaction. The luminescent readout is also unaffected by fluorescence interference from test compounds or other reaction components. A particularly beneficial feature of bioluminescent assays for profiling applications is the single luminescent readout. Several different enzyme assays can be combined on a single multiwell plate or in a single experimental run. This can facilitate data tracking and increase sample throughput by shortening the total read time compared to combining several assays that require different modes of detection or different filter sets, as is usually the case with fluorescent assays.

P450-Glo™ and MAO-Glo™ Assays are both luciferin-based and used to monitor drug effects on CYP450 and monoamine oxidase enzymes, respectively (2–4). A schematic describing the principle of the assays is shown in Figure 1. Here we present potency data for known inhibitors



**Figure 1. Conversion of luminogenic substrate by metabolic enzymes.** CYP450 or MAO enzymes act on a luminogenic substrate (Reaction A) to produce a luciferin product that generates light when the P450-Glo™ or MAO-Glo™ Reagent (Reaction B) is added after the metabolic enzyme reaction is complete. CYP450 and MAO selectivity depend on the nature of R<sub>1</sub> and R<sub>2</sub>.

of CYP450 and monoamine oxidase A (MAO-A) using this parallel approach and testing compounds in a dose-response format. First, we describe a completely automated system using Tecan instrumentation in a 384-well format. Next, we use the Labcyte Echo and the Deerac Fluidics Equator™ instruments for profiling of compounds in a 1536-well format. Both applications demonstrate the utility of the P450-Glo™ luminescent CYP450 assays<sup>(a,b)</sup> and MAO-Glo™ luminescent MAO assays<sup>(a,b,c)</sup> for generating inhibitory profiles for test compounds. Assays for MAO-A and the CYP450 enzymes CYP1A2, CYP2C9, CYP3A4, CYP2D6 and CYP2C19 are evaluated. These enzymes are important in the metabolism of drugs and other xenobiotic compounds.

### Fully Automated Metabolism Profiling

Automation plays a central role in screening facilities. The number of "hits" resulting from a screening campaign may require automated processing for confirmatory studies and secondary ADME testing. Complete walkaway systems allow

# Compound Profiling

Table 1. Z'-Factor Results for 384-Well and 1536-Well Metabolism Assays.

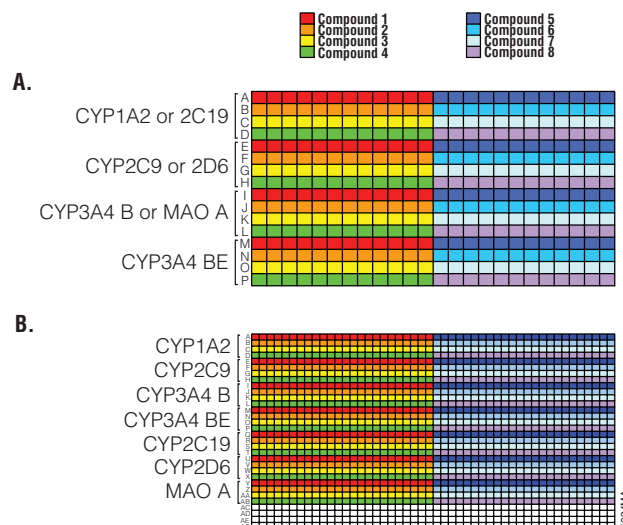
Assay	384-well	1536-well
CYP1A2	0.75	0.74
CYP2C9	0.71	0.74
CYP3A4*	0.83	0.79
CYP3A4**	0.80	0.78
CYP2C19	0.83	0.76
CYP2D6	0.79	0.83
MAO A	0.95	0.84

**384-well assays:** The Tecan TeMo™ was used to dispense enzyme and no-enzyme controls to a 384-well assay plate (Costar® Cat.# 3705). Substrates and NADPH Regenerating System (for the CYP450 reactions) were then added, followed by a 60-minute room-temperature incubation. P450-Glo™ and MAO-Glo™ Reagents were added for each assay, and luminescence was recorded following a 20-minute incubation. The 3A4 assays were conducted using two different substrates. **1536-well assays:** The Deerac Fluidics Equator™ was used to dispense enzyme and no-enzyme controls to a 1536-well assay plate (Costar® Cat.# 3937). Substrates and NADPH Regenerating System (for the CYP450 reactions) were then added, bringing the total reaction volume to 5µl. A 60-minute room-temperature incubation was then performed. Five microliters of P450-Glo™ or MAO-Glo™ Reagent was added for each assay, and luminescence was recorded following a 20-minute incubation. \*Prototype CYP3A4 substrate (substrate-B) was used. \*\*Luciferin-BE was used.

streamlined testing of these compounds, without the need for human intervention at any point within the process. In collaboration with Tecan AG Corporation, we developed a metabolism profiling application to demonstrate a completely automated solution for ADME screening in a 384-well format. By combining the P450-Glo™ and MAO-Glo™ Assay technology with flexible and sensitive liquid-handling and detection instrumentation, fully automated metabolism profiling of test compounds was easily achieved.

Prior to automating metabolism profiling with test compounds, assays were first tested for robustness and suitability for high-throughput screening (HTS) by performing Z'-factor measurements (5). Z'-factor value is a measure of the dynamic range and reproducibility of an assay. Z'-factors for all assays were above 0.7, indicating that the assay conditions were optimal for further profiling applications (Table 1).

A Tecan Freedom Evo® instrument with 8-tip LiHa, RoMa arm, integrated TeMo™ and Safire2™ plate reader was used for full automation of CYP450 and MAO assays. The plate layout in Figure 2, Panel A shows the placement of compounds and assays within the 384-well assay plates. Assay conditions were as described for Z'-factor measurements (Table 1).



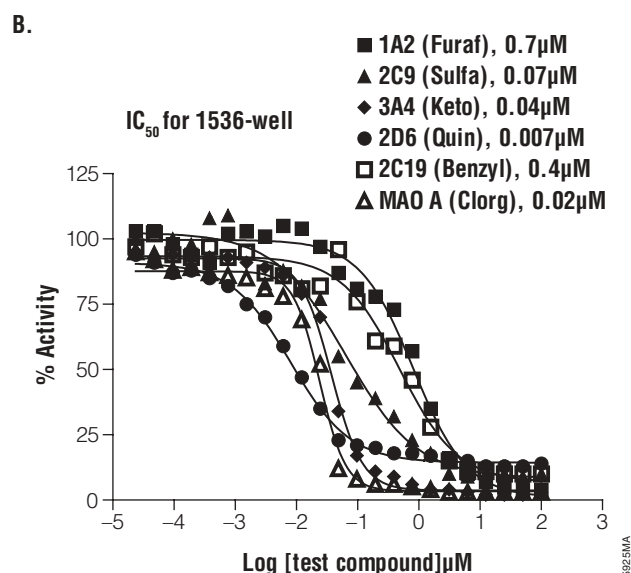
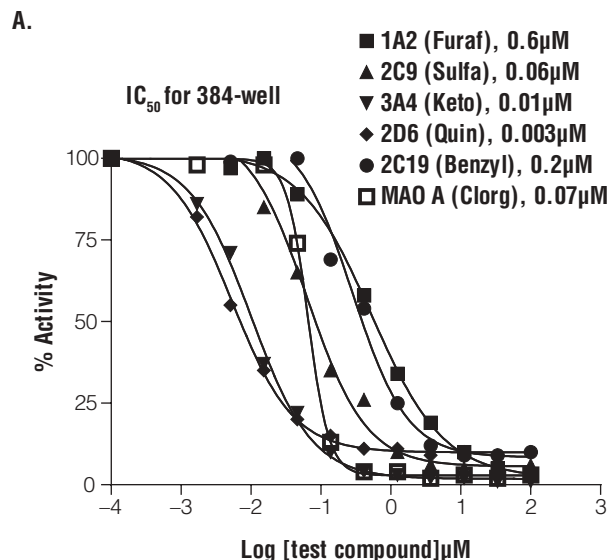
**Figure 2. Plate layouts for multiple assay metabolism profiling.** The plate layouts for both the 384-well (Panel A) and 1536-well (Panel B) profiling applications show the placement of test compounds and assays within each plate. For both plate formats, the colored areas represent the placement of test compound, with eight compounds tested per plate. A serial dilution of compound was performed within each plate, with one well per dose point. The dose response for each compound is arrayed from highest concentration to lowest, or DMSO-only control, from left to right. A 12-point dose response was performed in 384-well format (Panel A), and a 24-point dose response was performed in 1536-well format (Panel B). As indicated on both plate layouts, a different metabolism assay was performed in every four rows of the assay plate.

In a single run, seven assays for 32 test compounds were performed in parallel in a 384-well format, resulting in the generation of dose-response curves for every enzyme and compound combination where inhibition was observed. With seven enzyme assays performed for the 32 compounds tested, a total of 224 potency measurements were made in a single run. Tecan Magellan™ version 6.0 software was used to calculate IC<sub>50</sub> values. Figure 3 shows representative IC<sub>50</sub> curves for potent inhibitors. A summary of representative metabolism profiling data can be found in Table 2.

## Miniaturized Metabolism Profiling

Assay miniaturization is another means by which metabolism profiling can be performed. Miniaturization can save on the cost of reagent per reaction, but it also affords more data points per test compound at a comparable cost. We developed a 1536-well metabolism profiling application in collaboration with Labcyte and Deerac Fluidics as described in Figure 3. This 1536-well application demonstrates the possibility of performing metabolism assays in smaller volume formats.

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**Figure 3. Representative IC<sub>50</sub> curves from 384-well and 1536-well assays.** For the 384-well application, 12-point dose response curves were generated for each enzyme/compound combination. The final dose range of compound was from 2nM–100μM, with a no-compound control, serially titrated 1:3. For the 1536-well application, 24-point dose response curves were generated for each enzyme and compound combination. The final dose range of compound was 0.02nM–100μM, with a no-compound control, serially titrated 1:2. IC<sub>50</sub> results are comparable between 384-well and 1536-well formats. Each point represents one experiment per dose. Furaf = Furafylline, Sulfa = Sulfaphenazole, Keto = Ketoconazole, Quin = Quinidine, Benzyl = Benzylnirvanol, and Clorg = Clorgyline. **384-well fully automated assay method:** Reagent tubes were removed from their kit boxes and placed into either cooled or room-temperature tube holders on the deck of the Freedom Evo<sup>®</sup> instrument. Next, the 8-tip LiHa was used to assemble the various enzyme and substrate mixes required. A NADPH regenerating solution was also created for the CYP450 assays. All reagent mixes were assembled in an eight-row horizontally split trough and kept on a cooled transfer block on the deck of the Freedom Evo<sup>®</sup>. After reagent mixes were assembled, the TeMo<sup>™</sup> was used to add diluent to a 384-well compound source plate containing test compounds located in columns 1 and 13. The TeMo<sup>™</sup> then serially titrated DMSO solutions of the compounds 1:3 across the 384-well plate, ultimately creating a 12-point dose response curve (including no-compound control) with 1% final DMSO per reaction. Next, the TeMo<sup>™</sup> was used to array the diluted test compounds into assay plates. The RoMa arm then moved the reagent mixes from the deck of the Freedom Evo<sup>®</sup> to the TeMo<sup>™</sup>, where the TeMo<sup>™</sup> then assembled CYP450 and MAO reactions. Following a 60-minute incubation at room temperature, the TeMo<sup>™</sup> was used to add Luciferin Detection Reagent to the assay plates. After a 20-minute incubation with detection reagent, plates were moved with the RoMa arm to the Safire<sup>2™</sup> plate reader, and luminescence was recorded. **1536-well assay method:** 10mM test compounds (in 100% DMSO) were manually arrayed into a 1536-well Corning source plate. The Labcyte Echo was then used to dispense compounds into the 1536-well assay plate using acoustic droplet ejection. The Echo dispensed DMSO solutions of test compounds in various volume increments per well, ultimately achieving a 24-point dose response titration for each compound. In addition, the Echo backfilled 100% DMSO into each well, such that the final DMSO % for each reaction was 1%. Next, the Deerac Fluidics Equator<sup>™</sup> was used to dispense the remaining reaction components for the CYP450 and MAO assays, bringing the total reaction volume to 5μl. A 60-minute incubation was then performed. Five microliters of Luciferin Detection Reagent was added for each assay, and luminescence was recorded with the Tecan Safire<sup>2™</sup> plate reader following a 20-minute incubation.

We first generated Z'-factor data in a 1536-well format to test the robustness of the CYP450 and MAO assays in smaller reaction volumes. Z'-factors for all assays were above 0.7, indicating that the assay conditions were acceptable for further profiling applications (Table 1). We then developed a 1536-well metabolism profiling application. Figure 2 highlights the plate setup used for 1536-well metabolism profiling. On a single 1536-well plate, seven metabolism assays for eight test compounds were performed in parallel, resulting in the generation of IC<sub>50</sub> curves for every enzyme and compound combination. With seven assays per plate and eight test

compounds tested, a total of 56 potency determinations per 1536-well plate were generated. Figure 3 shows representative IC<sub>50</sub> curves for strong inhibitors. A summary of representative metabolism profiling data can be found in Table 2.

## Summary

Here we demonstrate that several metabolic enzyme assays can be performed in parallel by using a bioluminescent approach with a single readout. Thirty-two compounds were profiled in dose-response formats against seven enzyme activities in single automated experimental runs. The data

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Table 2. Representative IC<sub>50</sub> Profiling Data.

Compound/ Format	CYP1A2	CYP2C9	CYP3A4 B	CYP3A4 BE	CYP2D6	CYP2C19	MAO A
<b>Furafylline</b>							
384-well	0.6	>100	>100	NI	>100	NI	NI
1536-well	0.7	NI	40	NI	>100	NI	NI
Published values	0.67–6.0 (6–8)						
<b>Sulfaphenazole</b>							
384-well	NI	0.06	>100	NI	NI	NI	NI
1536-well	NI	0.07	>100	NI	NI	NI	NI
Published values	0.18–1.3 (6,8–11)						
<b>Ketoconazole</b>							
384-well	>100	1	0.01	0.02	13.6	1.5	>100
1536-well	>100	2.4	0.04	0.04	23	2.1	>100
Published values			0.083–0.17 (6,8,9,11,12)	0.083–0.17 (6,8,9,11,12)			
<b>Quinidine</b>							
384-well	NI	NI	>100	EC <sub>50</sub> 3.7	0.003	NI	NI
1536-well	NI	NI	>100	EC <sub>50</sub> 10.4	0.007	NI	NI
Published values	0.009–0.18 (6,8,9,11,12)						
<b>Nicardipine</b>							
384-well	6.9	0.04	0.3	0.4	1.6	0.3	NI
1536-well	10.6	0.03	0.34	0.39	1.4	0.52	NI
Published values	2.8–9.0 (13)						
<b>Deprenyl</b>							
384-well	8.3	NI	NI	NI	13.3	9.6	15.2
1536-well	14.9	NI	NI	NI	20	19.1	6.6
Published values	5* (14)						

This table highlights a subset of profiling data obtained from a total of 36 tested compounds. IC<sub>50</sub> values are listed for each enzyme and compound combination, unless otherwise noted. NI indicates no inhibition, where compound did not inhibit enzyme activity. >100: Test compound shows inhibition at higher doses, IC<sub>50</sub> is greater than the highest concentration tested. EC<sub>50</sub> indicates instances where the enzyme activity was stimulated by the compound. Values obtained from profiling assays are compared to those previously published in the literature for known inhibitors with the respective enzyme. Tecan Magellan™ v6.0 software was used to calculate IC<sub>50</sub>. \*indicates K<sub>i</sub> value.

show that known inhibitors generate inhibition profiles that agree with previously published values, and are comparable in both 384-well and 1536-well formats. The different inhibition profiles generated with the two 3A4 substrates suggests that there are substrate-dependent effects with the same compounds being tested (15).

From the profiling data generated here, it is clear that the same compound has varied affects on each metabolizing enzyme tested. Generating profiling data such as this early in the drug discovery process identifies inhibitory and occasionally stimulatory compounds. Performing multiple assays on one plate and simplified data tracking of compounds for each assay were made possible by the single luminescent readout. ■

# Compound Profiling

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## Protocols

*P450-Glo™ Assays Technical Bulletin #TB325*  
([www.promega.com/tbs/tb325/tb325.html](http://www.promega.com/tbs/tb325/tb325.html))

*P450-Glo™ Screening Systems Technical Bulletin #TB340*  
([www.promega.com/tbs/tb340/tb340.html](http://www.promega.com/tbs/tb340/tb340.html))

*MAO-Glo™ Assay Technical Bulletin #TB345*  
([www.promega.com/tbs/tb345/tb345.html](http://www.promega.com/tbs/tb345/tb345.html))

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## Ordering Information

Product	Size	Cat. #
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890
P450-Glo™ CYP1A1 Assay	10ml	V8751
P450-Glo™ CYP1B1 Assay	10ml	V8761
P450-Glo™ CYP1A2 Assay	10ml	V8771
P450-Glo™ CYP2C8 Assay	10ml	V8781
P450-Glo™ CYP2C9 Assay	10ml	V8791
P450-Glo™ CYP3A4 Assay	10ml	V8801
P450-Glo™ CYP3A7 Assay	10ml	V8811
P450-Glo™ CYP2C19 Assay	10ml	V8881
P450-Glo™ CYP2D6 Assay	10ml	V8891
MAO-Glo™ Assay	200 assays	V1401

Many of these products are available in additional sizes.

<sup>(a)</sup>U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.

<sup>(b)</sup>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.

<sup>(c)</sup>Patent Pending.

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