

A Fully Automated Platform For Drug Metabolism Profiling

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Abstract

Cytochrome P450s (CYP 450) and monoamine oxidases (MAO) are critical for the biotransformation of drug compounds. Adverse effects of test compounds on these enzymes often result in drug-drug interactions, leading to off-target levels of drugs within the body. Promega and Tecan have developed a complete automated solution for the profiling of test compounds in dose response format against CYP 450 and MAO. Superior luminescent metabolism assays for CYP 1A2, 2C9, 3A4, 2C19, 2D6 and MAO A, are combined with Tecan automation to bring a fully automated, walk away solution to the ADME screener.

Using a Tecan Freedom Evo® automated liquid handling instrument integrated with a Tecan Safire²™ plate reader, full automation of these metabolism assays in 384-well format includes: creation of reagent mixes, 12-concentration compound dilution, assay set-up, incubation and plate reading. In addition, Tecan Magellan™ software is used for automated data analysis. In a single run, a panel of seven metabolism assays for 32 test compounds is performed in parallel in 384-well format, resulting in the generation of dose response tests for every enzyme and compound combination. The single bioluminescent readout for the metabolism assays makes it possible to run multiple assays on one plate, simplifying plate reading, compound tracking, and data analysis. IC₅₀ results can be compared against all assays in the panel, allowing one to ascertain affects of their test compounds on the enzymes most involved in drug metabolism. The combination of Promega's luminescent assays with Tecan's hardware and software delivers validated results for metabolism testing.

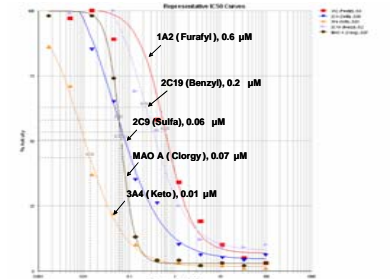
The Profiling Process

1. Enzyme/substrate and NADPH solutions (for P450 reactions) created with 8-tip LiHa.
2. Compound dilution and compound array into assay plates with TeMo™.
3. Assay assembly in assay plates with TeMo™.
4. Plate shake and incubation at room temperature for 1 hour.
5. Detection solutions added to each plate.
6. Plate reading with Safire²™.
7. Automated data handling with Magellan™ software.

Figure 2. An outline for automated profiling. Substrate, enzyme, NADPH components and assay buffers are placed directly from the kit boxes onto the deck of the Evo®. The 8-tip LiHa automatically assembles all enzyme/substrate and NADPH solutions required for the assays. Mixes are kept at 4°C on the deck until they are needed for the assay. Compounds are serially titrated across columns 1:3 by the TeMo™, and arrayed into 384-well assay plates (Costar #3705). The enzyme/substrate and NADPH solutions are moved to the TeMo™ with the RoMa arm, followed by the addition of solutions to the assay plate with the TeMo™. Plates are moved to a shaker with the RoMa, shaken and incubated for 1 hour at room temperature. Detection solutions are added to each plate, and plates are read following a 20-minute incubation. Automated data handling is performed with Magellan™.

Representative IC₅₀ Results

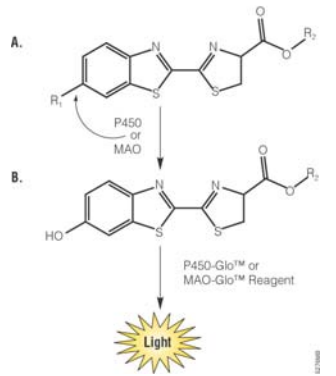
Figure 5. Representative IC₅₀ curves. Magellan™ data reduction software was used to determine potency for all enzyme and compound combinations. IC₅₀ curves for representative strong inhibitors are shown here. The final dose range of compound was from 2nM - 100µM, with a no-compound control, serially titrated 1:3. Each point represents one sample per dose.



Furafyl = Furafylline, Benzyl = Benzylnirvanol, Sulfa = Sulfaphenazole, Clorgyl = Clorgylline, Keto = Ketoconazole

The P450-Glo™ and MAO-Glo™ Assays

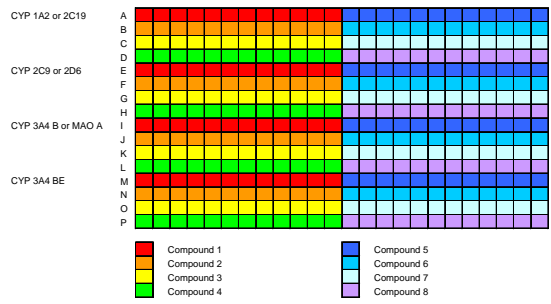
Figure 1. Conversion of luminogenic substrate by metabolic enzymes. CYP450 or MAO enzymes act on a luminogenic substrate (Reaction A) to produce a luciferin product that generates light when the P450-Glo™ or MAO-Glo™ Reagent (Reaction B) is added after the metabolic enzyme reaction is complete.



CYP450 and MAO selectivity depend on the nature of R₁ and R₂. Luciferin accumulation is directly proportional to light output. Therefore, light output is directly proportional to enzyme activity.

384-Well Assay Plate Layout

Figure 3. Compound and assay layout in the 384-well plate. The colored areas represent the placement of test compound with eight compounds tested per 384-well plate. The 12-point dose response for each compound is arrayed from highest concentration to lowest concentration, or DMSO-only control, from left to right. A different metabolism assay is performed in every four rows of the assay plate. For this fully automated method, a maximum of four assays are performed per plate for every eight compounds tested. The CYP 3A4 assay is performed with either Luciferin BE provided, or with a prototype substrate, Luciferin B.



As configured here, one run will test 32 compounds in dose response fashion against seven assays, for a total of 224 dose response determinations on eight 384-well plates.

Representative Profiling Results

	CYP 1A2	CYP 2C9	CYP 3A4 (B)	CYP 3A4 (BE)	CYP 2D6	CYP 2C19	MAO A
Furafylline	0.6	>100	>100	NI	>100	NI	NI
Published values	0.67 - 6.0						
Sulfaphenazole	NI	0.06	>100	>100	NI	NI	NI
Published values		0.18 - 1.3					
Ketoconazole	>100	1.0	0.01	0.01	13.6	1.5	>100
Published values			0.083 - 0.17	0.083 - 0.17			
Quinidine	NI	>100	>100	+	0.003	>100	NI
Published values					0.009-0.18		

Table 1. Representative profiling data. This table highlights a subset of data obtained from the profiling of 32 test compounds with the fully automated method described here. Values listed are IC₅₀ in µM, unless otherwise noted. NI indicates no inhibition, where compound did not inhibit enzyme activity. >100 indicates a test compound that shows inhibition at higher doses, and IC₅₀ is greater than the highest concentration tested (100µM). Values obtained from profiling assays are compared to those previously published in the literature for known inhibitors with the respective enzymes.

Instrumentation For Full Automation



Tecan Freedom Evo® with 2 meter deck, 8-tip LiHa, and RoMa arm

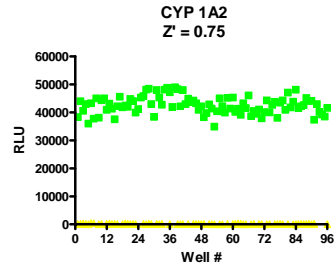
TeMo™ Multichannel Pipetting System

Freedom Evo® Standard Software

Safire²™ Multifunctional Plate Reader

Magellan™ Reader Control and Data Reduction Software

Z'-Factor



Assay	Z'-Factor
CYP 1A2	0.75
CYP 2C9	0.71
CYP 3A4 (B substrate)	0.83
CYP 3A4 (BE substrate)	0.80
CYP 2D6	0.79
CYP 2C19	0.83
MAO A	0.95

Figure 4. Z'-Factor results using Tecan automation. The Tecan TeMo™ was used to dispense enzyme and no-enzyme controls to the 384-well assay plate (Corning #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. Z'-factor is a measure of the dynamic range and reproducibility of an assay. Z'-factors for all assays were above 0.7 (0.5 is considered an excellent assay for HTS applications). Zhang, et al. (1999) Journal of Biomolecular Screening V4, No. 2, 67-73.

Summary

- P450-Glo™ and MAO-Glo™ Assays are designed for high throughput profiling applications.
- Performing multiple assays on one plate and simplified data tracking of compounds for each assay were made possible by the single luminescent readout for both assay technologies.
- Data presented here show known inhibitors generate inhibition profiles that agree with previously published values.
- From the profiling data generated here, it is clear that the same compound has varied effects on each metabolizing enzyme tested.
- Generating metabolism profiling data early in the drug discovery process identifies inhibitory and stimulatory compounds.
- Incorporating full automation for metabolism profiling allows for walk-away, accurate testing of compounds against metabolism enzymes involved in the biotransformation of drugs.