

# Miniaturized Luminescent Metabolism Profiling Assays

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## 1. Abstract

Cytochrome P450 and monoamine oxidases are two groups of enzymes involved in the metabolism of drugs. The interactions of these enzymes and compounds are studied carefully in early drug discovery process to facilitate greater success in clinical trials. Promega luminescent metabolism assays consist of the P450-Glo™ Screening Systems for CYP 1A2, 2C9, 3A4, 2C19 and 2D6, as well as the MAO-Glo™ Assay System for monoamine oxidase A. Using the Labcyte® Echo™ 550 acoustic liquid handler and the Deerac Fluidics Equator HTS reagent dispenser, a collection of compounds was profiled by IC<sub>50</sub> against the panel of metabolism assays in 1536-well format. Total assay volume of 5 µL was achieved by combining the acoustic technology and non-contact spot-on™ technology. Profiling with IC<sub>50</sub> determined potency of inhibition against selected enzymes involved in the drug metabolism process. The exceptional Z'-factor scores show the flexibility and reliability of Promega assays in conjunction with the Labcyte Echo and the Equator HTS from Deerac Fluidics in a high-throughput situation.

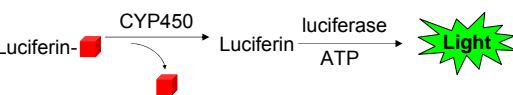
## 2. Introduction

Drug metabolism is an important aspect of pharmacokinetic studies because it is crucial for the elimination of drugs from the body. Drug-drug interactions can result when compounds either inhibit or induce the activity of enzymes necessary for metabolism, leading to off-target therapeutic levels of drugs within the body.

For this application, we focused on the testing of key enzymes involved in phase I biotransformation of drugs: cytochrome P450s and monoamine oxidases. In particular, cytochrome P450 (CYP450) isoenzymes 1A2, 2C9, 3A4, 2D6 and 2C19 are of interest due to their predominant contribution to the metabolism of drugs. Second to CYP450 are monoamine oxidases, in particular monoamine oxidase A (MAO A).

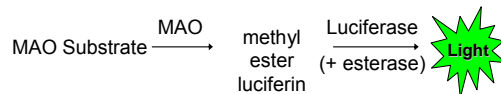
We demonstrate that by using assays with a single luminescent readout, metabolism enzymes can be run in parallel and profiled on one plate. Using sophisticated automation for compound dispensing and reagent additions, the profiling process can be miniaturized in 1536-format to decrease reaction volume and increase the number of data points for each test compound and assay combination.

## 3. P450-Glo™ Screening Systems



**Figure 1. Reaction scheme for P450-Glo Screening Systems.** The P450-Glo Assay Systems use modified luciferin substrates that when oxidized by CYP450, generate luciferin. Upon addition of a Luciferin Detection Reagent, luciferin reacts with ATP and O<sub>2</sub> to create light. The amount of light generated is directly proportional to the amount of luciferin created in the P450 reaction. Therefore, light output is proportional to CYP450 activity. P450-Glo can be used to screen new chemical entities for their capacity to modulate CYP450 activity. For this application, CYP450 isoforms 1A2, 2C9, 3A4, 2D6 and 2C19 were studied. Two different substrates for 3A4 (substrate B prototype and substrate BE) were also evaluated.

## 4. MAO-Glo™ Assay System



**Figure 2. Reaction scheme for MAO-Glo Assay.** The MAO enzyme oxidizes the amine of the aminopropylether luciferin analog to an imine. Following spontaneous β-elimination, a methyl ester is formed. A Luciferin Detection Reagent is added which simultaneously inactivates the MAO enzyme, and the esterase and luciferase enzymes in the reagent hydrolyze the methyl ester and oxidize luciferin to produce light. The amount of light produced is directly proportional to MAO activity. MAO-Glo can be used to screen new chemical entities for their capacity to modulate monoamine oxidase activity. The MAO A enzyme was studied for this application, although the assay can be used for either MAO A or MAO B.

## 5. Labcyte® Echo™



The Echo 550 and Echo 555 liquid handling systems use sound to transfer compound in droplets of 2.5nl. No pipette tip or pin tool ever touch the samples during transfer.

### Acoustic drop ejection improves IC<sub>50</sub> analyses in a number of ways:

- Acoustic drop ejection requires dramatically less sample. Compounds tested were stored in Echo qualified 1536-well source plates (Labcyte # LP-03730), 5µl of 10-50mM per well.
- The elimination of serial aqueous dilutions improves IC<sub>50</sub> values, reduces false negative results, lowers consumable cost, and reduces plastic and reagent waste.
- Acoustic droplet ejection has high precision (<8% CV at 2.5nl) and eliminates accumulated error.

**Figure 3 (From top to bottom):** Using sound energy, Labcyte Echo 550 ejects drops of sample directly from source plate to assay plate.

## 6. Deerac Fluidics™ Equator™



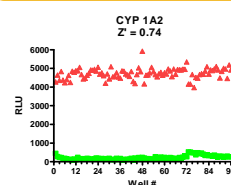
The Deerac Fluidics Equator HTS eight-tip pipetting system is designed for assay development and high throughput screening applications. The system dispenses volumes ranging from 20µl down to 50nl in 96, 384, and 1536-well plate formats using on-the-fly pipetting technology. The system channels can all individually aspirate and dispense varying volumes with high accuracy and reproducibility.

### Key Features of the Equator HTS

- Aspirate dispense system with low dead volume
- 8-Channel pipetting
- 20µl to 50nl dispense range
- Independent channel control for dispensing gradients and regions

**Figure 4. The Equator HTS is a flexible instrument for both assay development and screening applications.**

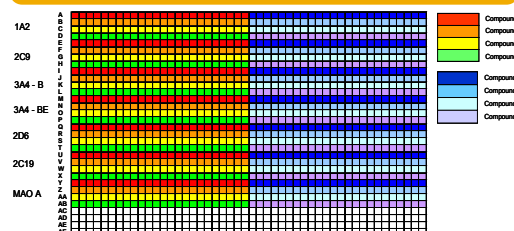
## 7. Z'-Factor



Assay	Z'-Factor
CYP 1A2	0.74
CYP 2C9	0.74
CYP 3A4*	0.79
CYP 3A4**	0.78
CYP 2D6	0.83
CYP 2C19	0.76
MAO A	0.84

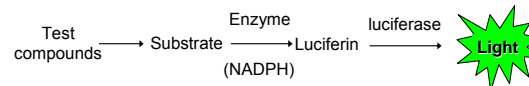
**Figure 5. Z'-factor results.** Reactions were conducted in a 5µl volume and incubated at room temperature for one hour. 5µl of detection reagent was added, followed by a 20-minute room temperature incubation. Luminescence was recorded with the Tecan® Safire™ plate reader. Z' was > 0.7 for all assays, indicating the reaction conditions were suitable for further experiments. \* = 3A4 substrate B prototype. \*\* = 3A4 substrate BE. Reference : Zhang, *et al.* (1999) *J Biolom Screen* 4 (2), 67-73.

## 8. 1536-Well Profiling Plate Layout



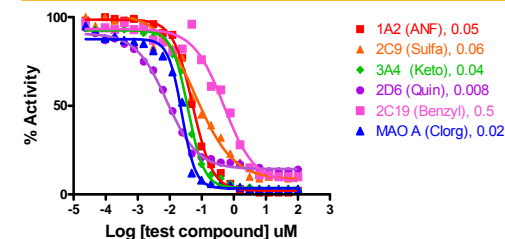
**Figure 6. 1536-well plate layout.** Eight different compounds were tested for each assay in 24-point dose response format (n = 1 per dose), with each color indicating the placement of each compound titration within the assay plate. Compounds were titrated 1:2 from left to right, ranging from 100µM to 0µM in each assay. Seven different assays were performed on each plate as indicated above for a total of 56 dose response tests per plate using this layout.

## 9. Profiling Process



1. Use Labcyte Echo to prepare compound dose response curves and DMSO back fill to bring DMSO volume to 50nl per well (1% DMSO final in assay). Final concentration of compound = 100 µM to 0.02nM; DMSO-only wells are used as controls.
2. Dispense substrates, enzymes, and NADPH (for P450 reactions) with Deerac Fluidics Equator (4.95µl total between volume additions).
3. Incubate 1-hour at room temperature.
4. Add Luciferin Detection Reagents with Deerac Fluidics Equator (5µl addition).
5. Incubate 20 minutes at room temperature.
6. Record luminescence with Tecan Safire<sup>2</sup> plate reader.

## 10. Representative Titration Results



**Figure 7. Representative IC<sub>50</sub> data.** The IC<sub>50</sub> results here (in µM concentration) are representative titration results obtained with strong enzyme inhibitors. Results are comparable (within 5-fold) to those reported in literature. ANF = alpha-Naphthoflavone, Sulfa = Sulfaphenazole, Keto = Ketoconazole, Quin = Quinidine, Benzyl = Benzylnirvanol, Clorg = Clorgyline.

## 11. Representative Profiling Data

	CYP 1A2	CYP 2C9	CYP 3A4 (B)	CYP 3A4 (BE)	CYP 2D6	CYP 2C19	MAO A
Furafylline	0.7	NI	> 100	NI	>100	NI	NI
Sulfaphenazole	0.67-6.0	0.06	17.5	NI	NI	NI	NI
Ketoconazole	14.5	2.4	0.04	0.04	23	2.1	> 100
Quinidine	NI	NI	0.083-0.17	0.083-0.17	0.008	NI	NI
Published values					0.009-0.18		

**Table 1. Representative profiling results.** This table highlights a subset of profiling data obtained from the 36 compounds tested. Results are listed as IC<sub>50</sub> in µM unless otherwise noted. + = positive cooperativity where the test compound stimulated activity of the respective enzyme. NI = no inhibition of the test compound with the respective enzyme. >100 = inhibition at higher doses, and IC<sub>50</sub> is greater than the highest concentration tested (100µM).

## 12. Summary

- Luminescent metabolism profiling assays can be performed on a single plate, with only one endpoint readout needed to capture data.
- Performing multiple assays on the same plate allows for simultaneous enzyme analysis, simplifying data and sample tracking.
- Miniaturization enables more data points per plate.
- The Labcyte Echo delivers precise volumes of test compound, indicated by accurate curve fitting and comparable IC<sub>50</sub> results to literature values.
- Using acoustic dispensing of compound kept the % DMSO low in each assay to avoid interference with results.
- The 8-channel pipetting capability of the Deerac Fluidics Equator made it possible to set-up a different assay per channel.
- The acoustic dispensing, small-volume pipetting, and robust assays make metabolism profiling in 1536-well format a reality.