Automating Cytochrome P450 Screening Using Promega P450-Glo™ CYP450 Assays and the Protedyne BioCube™ System

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Abstract

Many drug discovery programs incorporate high throughput screening of cytochrome P450 activity to identify potential drug toxicities and improve the efficiency and cost effectiveness of the drug development process. In this investigation, Promega P450-Glo kits for CYP1A1, CYP1A2, CYP2C9, CYP2C19*, CYP2D6*, and CYP3A4 were evaluated on the Protedyne BioCube System, an industrial grade automation platform configured with a BMG Labtech PheraStar reader. Each enzyme was subject to the following tests performed in parallel manually and on the automated BioCube System: IC_{50} determinations, compound screening with known inhibitors, Z'- Factor, and limit of detection. IC_{50} determinations and compound screening exhibited good correlation between manually generated and BioCube System-generated inhibition values. Z'- Factor scores ranged from 0.69 to 0.79 revealing robust performance with good dynamic range of the automated assay. Limit of detection values ranged from 0.97 to 7.8 fmol of enzyme highlighting the sensitivity of the assays.

* Assays currently under development

Introduction

Cytochrome P450 enzymes play a pivotal role in the metabolism of drug compounds. Compromising the normal activity of these enzymes results in toxicity. It is critical that adverse effects of drug compounds be detected at an early stage to reduce attrition of toxic compounds in later stages of drug development. High throughput automated screening assays for cytochrome P450 activity are essential for the rapid identification of problematic compounds.

Protedyne and Promega have partnered to demonstrate a high throughput application of Promega's P450-Glo™ Assays on the Protedyne BioCube System, providing an integrated solution combining chemistry and hardware to meet the needs of today's screening laboratory.

Figure 1. Picture of BioCube System LX2000 with an Input 256 Plate Carousel and right-side Incubator

Materials

Hardware

Assays were run on the BioCubeSystem automation platform. The enclosed system consists of:

- 256 Plate Carousel, Ambient Temperature
- 4-Axis Robot Arm with On-the-Fly Changeable Tools Using SmartTool™ Technology
- Plate Gripper SmartTool
- 20 µl, 96-Channel Pipettor SmartTool
- 200 µl, 96-Channel Pipettor SmartTool
- Variomag Teleshake-70
- BMG Labtech PheraStar Reader*

The system performed all steps of the protocol, with the exception of loading the plates into the carousel, placing the reagent troughs on the deck prior to starting the protocol, and data analysis. The robot arm automatically changed tools for plate and liquid handling as required by the protocol.

* Not located within enclosed system

Software

The system uses an Adept robot controller running Protedyne's CILA™ system software. The protocol was programmed off-line using CILA-View application development software.

Labware

- 384 well Whatman, white, flat-bottom plate **(assay plate)**
- 96 well Costar, white, round-bottom plate **(compound plate)**
- 96 well Phenix PCR plate **(reagent plate)**

Figure 2. The BMG Labtech PHERAstar microplate reader. The PHERAstar is a multifunctional plate reader with a variety of detection modes including luminescence, fluorescence intensity, fluorescence polarization, and absorbance. For this application, the PHERAstar was used to read plates in 384-well density.

Methods

Z'- Factor Test

- 6.25 µl of luciferin-free water and 12.5 µl of 2X enzyme/substrate were dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 10 minutes at room temperature.
- 6.25 µl of 4X NADPH regeneration system was then dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 60 minutes at room temperature.
- 25 µl of luciferin detection reagent was dispensed to the assay plate and subsequently shaken for 10 seconds at 1200 rpm, incubated for 20 minutes at room temperature followed with analysis in the BMG Labtech PheraStar reader.

Compound Screen Test

- 6.25 µl of compounds (10µM final concentration) and 12.5 µl of 2X enzyme/substrate were dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 10 minutes at room temperature.
- 6.25 µl of 4X NADPH regeneration system was then dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 60 minutes at room temperature.
- 25 µl of luciferin detection reagent was dispensed to the assay plate and subsequently shaken for 10 seconds at 1200 rpm, incubated for 20 minutes at room temperature followed with analysis in the BMG Labtech PheraStar reader.

Limit of Detection Test

- 40 µl enzyme (2 pmol final concentration)/substrate was serially diluted (1:2) twelve times in 40 µl diluent.
- 12.5 µl of 2X enzyme/substrate and 6.25 µl of luciferin-free water were dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 10 minutes at room temperature.
- 6.25 µl of 4X NADPH regeneration system was then dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 60 minutes at room temperature.
- 25 µl of luciferin detection reagent was dispensed to the assay plate and subsequently shaken for 10 seconds at 1200 rpm, incubated for 20 minutes at room temperature followed with analysis in the BMG Labtech PheraStar reader.

IC50 Test

- 20 µl compound (varying final concentrations) was serially diluted (1:3) twelve times in 40 µl diluent.
- 6.25 µl of compound and 12.5 µl of 2X enzyme/substrate were dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 10 minutes at room temperature.
- 6.25 µl of 4X NADPH regeneration system was then dispensed to the assay plate and subsequently shaken for 20 seconds at 1200 rpm then incubated for 60 minutes at room temperature.
- 25 µl of luciferin detection reagent was dispensed to the assay plate and subsequently shaken for 10 seconds at 1200 rpm, incubated for 20 minutes at room temperature followed with analysis in the BMG Labtech PheraStar reader.

Figure 3. The P450-Glo bioluminescent reaction. In the P450-Glo Assays, modified luciferin substrates are cleaved by the specific cytochrome P450, releasing luciferin which is then free to react with the firefly luciferase enzyme, ATP and oxygen to produce light. Light generated from this reaction is proportional to cytochrome P450 enzyme activity. Signal stability of the luminescence is > 2 hours, making this assay amenable to batch processing.

1A1	Manual %Inhibition	BioCube %Inhibition
ANF	99	99
Phenacetin	41	38
Quinidine	77	73
1A2		
ANF	99	99
Fluvoxamine	98	98
Furafylline	89	90
2C9		
Diclofenac	88	82
Nitrendipine	96	91
Sulfaphenazole	97	93
2C19		
Troglitazone	98	73
Fluvoxamine	95	91
Isoniazid	6	5
2D6		
Terfenidine	84	85
Quinidine	89	91
Nicardipine	89	67
3A4		
Erythromycin	41	28
Ketoconazole	90	93
Troleandomycin	84	85
Verapamil	68	76

Table 2. 10µM compound screen. Three to four known compound inhibitors were screened against each CYP450 enzyme. Percent inhibition was determined relative to a vehicle control using Excel.

Enzyme	Z' Score	CV
1A1	0.74	8.7%
1A2	0.79	6.7%
2C9	0.69	9.5%
2C19	0.77	7.5%
2D6	0.74	8.6%
3A4	0.79	6.5%

Table 1. Z'- Factor Score for the P450-Glo Assays. Each cytochrome P450 was evaluated in relation to Sf9 control. The Z'- Factor was calculated using the following equation in Excel:
1-(3*SD enzyme + 3*SD Sf9 / | Mean enzyme – Mean Sf9|)

Summary

- The BioCube System can perform P450-Glo Assays in an equivalent manner to manual procedures.
- The Z' Factor scores were well above 0.5, demonstrating robust performance of the assay on the Protedyne BioCube automated platform.
- Results from the compound screen show strong correlation between the manual and BioCube methods.
- IC_{50} showed 100% correlation between manual and BioCube methods for all compounds tested.
- The BioCube System can process 135 assay plates in an 8 hour time frame.

References

1. Zhang et al. (1999) Journal of Biomolecular Screening. V 4, No. 2, 67-73.
2. P450 Glo technical bulletin No. 325.

1A1	Manual IC_{50}	BioCube IC_{50}
Phenacetin	15.96 µM	39.8 µM
Quinidine	3.12 µM	5.8 µM
1A2		
ANF	0.05 µM	0.15 µM
Fluvoxamine	0.11µM	0.44 µM
2C9		
Diclofenac	2.1 µM	1.5 µM
Sulfaphenazole	0.06 µM	0.03 µM
2C19		
Fluvoxamine	0.28 µM	0.65 µM
Isoniazid	88.7 µM	115 µM
2D6		
Quinidine	10 nM	10 nM
3A4		
Ketoconazole	0.01 µM	0.05 µM
Troleandomycin	0.46 µM	0.66 µM
Verapamil	0.53 µM	0.31 µM

Table 3. IC_{50} determination. Test compounds were serially diluted on the BioCube System. IC_{50} was calculated using GraphPad Prism V4.0 software.

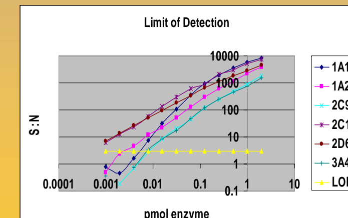

Figure 4. Limit of detection for the P450-Glo Assays. Each cytochrome P450 was serially diluted on the BioCube System to determine assay sensitivity. Signal to noise ratio determined relative to Sf9 control using Excel.