

# Automation of a Multiplexed Cell-based Assay to Measure Simultaneously Inhibition and Induction of the Cytochrome P450 Isoform 3A4 by Small Molecule Compounds



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## Overview

The role that CYP3A4 plays in the metabolism of drugs has been well documented. Therefore, it is essential to understand how this P450 isoform is affected by xenobiotics, to avoid possible drug-drug interactions. Compounds have been shown to either inhibit the activity of CYP3A4 or induce its expression. What is less understood is how a drug can induce gene expression, and inhibit activity of the same isoform. Here we demonstrate the automation of a cell-based multiplexed assay which provides this information. Inhibition and induction were measured using a luminogenic, cell-membrane permeable 3A4-specific substrate and a luciferin based reporter gene assay, respectively. Normalization to cell number was also performed using a fluorescent cell viability assay. DPX-2 cells were dosed for 48 hours with variable concentrations of 54 individual compounds. The combined information from the triplex assay provides a comprehensive picture of a drug's effects on CYP3A4.

## Introduction

The Pregnane X Receptor (PXR) is a nuclear receptor which plays a part in the removal of foreign substances from the body. Upon detecting these xenobiotics, PXR responds by upregulating proteins that can help in the elimination process. One of the most important enzymes involved in the response is the cytochrome P450 3A4 enzyme. CYP3A4 is involved in the metabolism of the widest range of substrates, and metabolizes more than 50% of drugs on the market today. PXR upregulates the expression of this enzyme by binding to the response element of the promotor.

Many drugs upregulate the expression of the CYP3A4 gene via PXR. In contrast, a large set of drugs have also shown inhibitory effects on the CYP3A4 enzyme. The upregulation of the CYP3A4 gene or inhibition of the enzyme can result in adverse drug-drug interactions. Less understood and more difficult to assess is how certain drugs can induce the expression of the CYP3A4 gene and also inhibit the activity of the enzyme. The DPX2 cell line is a useful tool to assess the effects of a xenobiotic on gene induction and enzyme inhibition. DPX2 cells contain human PXR and a luciferase-linked CYP3A4 promoter reporter construct. Upon activation, PXR binds to the native promoter and the luciferase-linked promoter. Increased CYP3A4 enzyme expressed from the native gene, as well as luciferase expressed from the reporter construct is then expressed. Activity of the enzyme may or may not be inhibited, depending on the effects of the drug. We have used a triplex assay with DPX2 cells to assess the full effects of set of drugs on CYP3A4. The assay was automated in 96-well format using two instruments ideally suited to dispense cells, titrate compounds, transfer and remove media, and dispense compounds and reagents to the assay plates. A multi-mode, monochromator-based reader was then used to read the luminescent and fluorescent signals from the plates. The combined cell line, assay, and instrumentation create the ideal solution to assess CYP3A4 expression and activity.

## BioTek Instrumentation

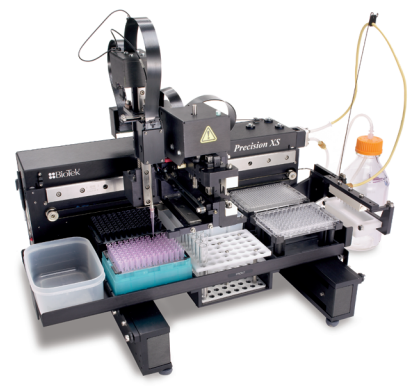


Figure 1 – Precision™ XS Microplate Sample Processor

The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dispense cells to TC treated plates, serially titrate compounds across a 96-well PP plate, transfer compounds to the cell plates and media from the cell plates to luminometer plates, as well as dispense assay reagents to all 96-well plates.



Figure 2 – EL406 Combination Washer Dispenser

The EL406 offers fast, accurate media removal and plate washing capabilities through its Dual-Action™ Manifold. It also offers reagent dispensing capabilities through the use of its peristaltic or syringe pumps, with volumes ranging from 1-3000 µL/well. The instrument was used to remove media, as well as dispense Luc-IPA substrate in media to the 96-well cell plates.



Figure 3 – Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader

The Synergy™ Mx incorporates a quadruple monochromator system which selects wavelengths between 230 and 900 nm in 1 nm steps. The system includes variable band pass selections, incubation up to 65°C and advanced data analysis software. The instrument was used to measure luminescent and fluorescent signals.

## Synergy™ Mx Settings

Synergy Mx Optimized Instrument Settings		
Setting	Fluorescence	Luminescence
Detection Method	Fluorescence	Luminescence
Excitation Setting	400/20 nm	NA
Emission Setting	505/20 nm	NA
Optics Position	Top	Top
Delay after Plate Movement	100 msec	100 msec
Measurements per Data Point	10	NA
Integration Time	NA	1.0 Sec
Top Probe Vertical Offset	8.00 mm	1.00 mm
Dynamic Range	NA	Extended
Auto Scale Automatic Sensitivity Adjustment Used		

Table 1 – Synergy™ Mx Reader Settings

## DPX2 Cell-Based Triplex Assay Automated Procedure

A total of 54 compounds were tested in the study. Seven compounds were run per plate, plus a control compound (Rifampicin), creating eight 96-well plates tested. The final plate contained four test compounds plus the control. Four plates were run in two separate groups, with staggered starting dates. The procedure below explains the steps taken to assay the compounds in one set of four plates.

### Day 1

1. Dispense 20K DPX2 cells in Puracyp Culture Media from a trough to columns 1-11 of the Corning white, 96-well TC treated plates (Corning Part #3903) using the Precision™ XS. Incubate the cells overnight in a 37°C, 5% CO<sub>2</sub> incubator.

### Day 2

1. Perform 11-point 1:3 compound titrations (50-0 µM), by serially transferring 75 µL of diluted compounds into 150 µL of Puracyp Dosing Media, using the Precision™ XS.
2. Remove the media from the cell plates using the manifold on the EL406.
3. Transfer 100 µL from column 11 of the titration (no compound) to column 11 of the cell plate, using the Precision™ XS. Using the same tips, repeat the transfer moving from right to left on each plate. Place the cell plates back into the 37°C, 5% CO<sub>2</sub> incubator, and incubate overnight.

Repeat this procedure for the number of plates included in the test.

### Day 3

1. Add fresh compound to the cell plates by repeating the process outlined for day 2.

### Day 4

1. Remove the media from the cell plates using the manifold on the EL406. Dispense 100 µL of 3 µM Luc-IPA in Puracyp Dosing Media to all wells of the plates, using the peristaltic pump on the EL406. Place the cell plates back into the 37°C, 5% CO<sub>2</sub> incubator, and incubate for 60 minutes.
2. Transfer 60 µL of the media in each well of the cell plates to a new Corning white, 96-well luminometer plate (Corning Part #3912) using the Precision™ XS.
3. Add 60 µL of Luciferin Detection Reagent (Promega Part #V859 and V144) to the 96-well luminometer plates using the Precision™ XS.
4. Shake the plates for 30 seconds on an orbital shaker, incubate at RT for 20 minutes, and read using the luminescent mode on the Synergy Mx.
5. Add 10 µL of 5X CellTiter-Fluor™ Cell Viability Assay (Promega Part #G6080) to the 40 µL of media remaining in the cell plates, using the Precision™ XS.
6. Shake the plates for 30 seconds on an orbital shaker, incubate in the 37°C, 5% CO<sub>2</sub> incubator for 30 minutes, and read using the fluorescence mode (Ex:400/Em:505) on the Synergy Mx.
7. Add 50 µL of ONE-Glo™ Luciferase Assay System (Promega Part #E6110) to the cell plates containing media and CellTiter-Fluor™.
8. Shake the plates for 30 seconds on an orbital shaker, incubate at RT for 2-3 minutes, and read using the luminescent mode on the Synergy Mx.

## DPX2 Cell Triplex CYP3A4 Induction Assay

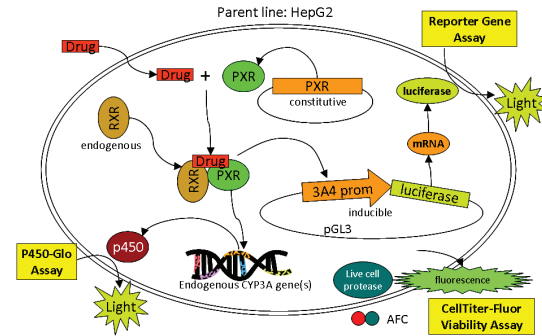


Figure 4 – Representation of the DPX2 cell-based Triplex assay

DPX2 cells contain human PXR and a luciferase-linked CYP3A4 promotor. Upon activation, PXR dimerizes with RXR and binds to the response element of the endogenous CYP3A4 promotor, causing increased expression of CYP3A4. The P450-Glo™ Assay with the Luc-IPA substrate detects the activity of the CYP3A4 enzyme. Following activation, PXR and RXR also bind to the response element of the introduced CYP3A4 promotor, causing increased luciferase production. Through the addition of the One-Glo™ reporter gene assay luminescent signal is detected that is proportional to CYP3A4 gene expression. CellTiter-Fluor™ reagent is also added to detect cell viability and normalize other assay results to cell number. A fluorogenic, cell-permeable, peptide substrate is added to the cells. Live cells cleave the substrate to create fluorescent AFC. Fluorescent signal is proportional to cell viability.

## Cell Plating and Compound Titration Verification

### Cell Plating

The ability of the Precision XS to accurately dispense cells was verified by analyzing the %CV of the CellTiter-Fluor™ values across wells from all 8 cell plates showing no compound viability effects. A second method was also used where %CV was computed for plate 1 rows showing no viability effects across the entire row. This was done to determine the normal variability seen for cell dispensing. Results were 4.6% and 5.0%, respectively, for the two methods, confirming the instrument's capability to dispense DPX2 cells in 96-well format.

### Plate 1

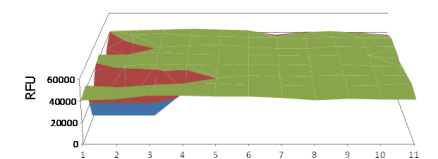


Figure 5 – Precision™ XS DPX2 cell dispensing accuracy using CellTiter-Fluor™ data

### Compound Titration

The Precision's ability to accurately titrate compounds was also verified. Rifampicin was run as a control compound on each plate, and the average and 95% confidence interval for all 8 EC<sub>50</sub> values was computed using the net P450-Glo™ signal (P450-Glo™ Signal – Avg(No Cell Control)). This value was 1.0 +/- 0.2 µM, which was within generally accepted variability for pharmacology. The consistency among control compound EC<sub>50</sub> values also gives confidence in each test plate's data. Even though P450-Glo™/CellTiter-Fluor™ ratios vary from plate to plate, due to small variances in raw values, the EC<sub>50</sub> values remain equivalent, showing that pharmacology is not affected by plate variation.

### DPX2 Multiplex EC<sub>50</sub> rifampicin positive control

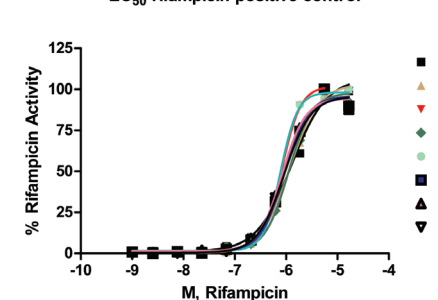


Figure 6 – Rifampicin EC<sub>50</sub> curves for test plates included in study. Y-axis represents the ratio of P450-Glo™ luminescent values over CellTiter-Fluor™ fluorescent values per well.

## Compound Induction/Inhibition Analysis

The set of 54 compounds was tested in dose response mode. CYP3A4 gene induction, enzyme activity, and cell viability was computed for each compound. One-Glo™ and P450-Glo™ fold induction over basal activity was used to determine the affect of the compound on CYP3A4 expression and enzyme activity. The latter was also used to analyze any inhibitory affects that the compound might have on enzyme activity. Three types of control compound were included in the test. (Data for non-control compounds to be published at a later date.) Rifampicin is a well known inducer of CYP3A4 gene expression and activity, and is included as the positive control<sup>1</sup>. Bergamottin has been shown in literature to induce CYP3A4 gene expression, while also inhibiting enzyme activity<sup>2</sup>. The result is increased fold induction in the One-Glo™ assay, and inhibition in the P450-Glo™ assay. Finally, Acetaminophen shows no affect on CYP3A4 gene expression or enzyme activity<sup>3</sup>. Therefore no increase over basal levels should be seen with either assay. Agreement with expected results was seen for all control compounds. The shape of the Bergamottin P450-Glo™ curve shows the complexity of the two counteractive influences within the cell. Inhibition by approximately 2-fold is seen at very low levels of compound, followed by over 2-fold induction at 5 µM. Enzyme activity is then once again inhibited at higher concentrations of compound.

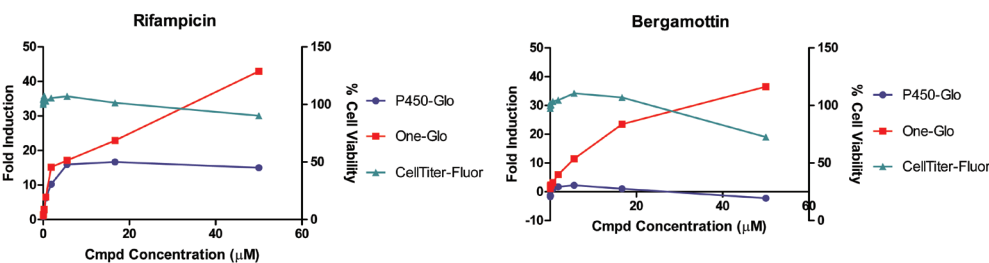


Figure 7 – Fold induction levels for control compounds

P450-Glo™ and ONE-Glo™ fold induction were computed as follows. The average of the no cell background was subtracted from the individual luminescent signals for that compound concentration. This value was then divided by the CellTiter-Fluor™ (CTF) signal from the same well. This created a normalized, background subtracted value for each compound containing well. The average of the no cell background subtracted basal luminescent signals, divided by the basal CTF signal was also computed. This created a normalized, background subtracted average basal signal. The compound containing normalized values were then divided by the average normalized basal signal, creating a fold induction value for each compound concentration over the basal level.

The CellTiter-Fluor™ % cell viability was computed by dividing the CTF signal for each compound concentration by the CTF basal signal.

## Conclusions

1. The Precision™ XS provides an easy-to-use, dependable solution for dispensing cells, titrating compounds, and delivering reagents to assay plates.
2. The liquid handling capabilities of the EL406's Manifold and Peristaltic Pump provide rapid and reliable media removal and reagent dispensing.
3. Promega's P450-Glo™, ONE-Glo™, and CellTiter-Fluor™ triplex assay provides a straightforward way to assess multiple compound affects a compound may have on a cell.
4. The combination of BioTek's instrumentation, and Promega's cell-based triplex assay create an ideal solution to help fully understand the stimulatory and inhibitory effects of a drug.

1. U.S. Department of Health and Human Services, FDA: Guidance for Industry Drug Interaction Studies Sept. 2006. 2. Wen YH, Sahi J, Urda E, Kulkarni S, Rose K, Zheng X, Sinclair JF, Cai H, Strom SC, Kostrubsky VE: Effects of bergamottin on human and monkey drug-metabolizing enzymes in primary cultured hepatocytes. *Drug Metabolism and Disposition: the biological fate of chemicals* Sept. 2002; 30(9) 977-84. 3. Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, Rodrigues AD: Evaluation of 170 Xenobiotics as Transactivators of Human Pregnane X Receptor (hPXR) and Correlation to Known CYP3A4 Drug Interactions. *Current Drug Metabolism* 2006; 7(4) 375-388