Automated Triplexed Hepatocyte-Based Viability and CYP1A and CYP3A Induction Assays in 96- and 384-well Microplates

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Overview

The ability to monitor CYP induction, inhibition, and cytotoxicity by xenobiotics using an automated, multiplexed format can decrease workloads and increase data confidence. Here we demonstrate the ability to monitor CYP1A and -3A induction, combined with a cytotoxicity measurement, from a single well using cryopreserved human hepatocytes. The assay procedure was automated in 96- and 384-well formats, including cell manipulations, compound titration and transfer, and reagent dispensing, using simple, yet robust robotic instrumentation. EC_{50} values were derived for multiple known inducers of CYP1A and -3A from an 11-point curve. Induction and toxicological responses in the triplex system were validated based on consistency with conventional single parameter assays. Validation and pharmacology data confirm that multiplexed cell-based CYP assays can simplify workload, save time and effort, and generate the data needed from today's ADME/Tox laboratory.

Introduction

Drug-drug interactions (DDI) are of serious concern to the pharmaceutical industry and associated regulatory agencies. One parameter of DDI is induction, the upregulation of enzyme levels that increase metabolism, reducing a drug's therapeutic efficacy. Aryl hydrocarbon receptor (AHR), constitutively active receptor (CAR) and pregnane X receptor (PXR) are nuclear receptors responsible for transcription of the majority of enzymes and transporters associated with drug metabolism and excretion. However, all the enzymes and transporters do not need to be measured. To quantify this risk, the FDA guidance states that in vitro CYP1A and CYP3A activities may be used to monitor induction potential of all drug metabolizing CYPs. Though there are cell lines and reporter gene assays that can be used, the gold standard is the use of hepatocytes to assess the induction potential of a drug by measuring the enzymatic activity of CYP1A and CYP3A with specific substrates and comparing the activity to the basal rate of the enzyme. Typically this information has been gathered from separate wells of cultured hepatocytes and by combiningthe data from multiple experiments. This process is time-consuming and labor intensive, and it may lead to misinterpretation when combining the data due to the variability within each assay. A multiplexed format provides a method to obtain multiple readouts from a single microplate well, which may attenuate these concerns.

Here we demonstrate the ability to monitor CYP1A and CYP3A induction, combined with cytotoxicity measurements, from a single well using cryoplateable hepatocytes. The assay procedure was automated in 96- and 384-well formats, including cell manipulations, compound titration and transfer, and reagent dispensing, using simple, yet robust robotic instrument. Four known inducers of CYP1A and four known inducers of CYP3A were used as control compounds to validate the triplex assay. Results show how the ability to measure three critical responses from a single sample streamlines work flow, derives maximum value from costly hepatocytes, and provides a means for reaching more biologically relevant conclusions.

BioTek Instrumentation

BioTek Liquid Handling

A. The EL406™ Combination Washer Dispenser 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 for cell dispensing, media exchange and removal, as well as dispensing of reagents to the cell plates. The small footprint of the instrument allows for easy insertion into existing laminar flow hoods to ensure sterile manipulations.

B. The Precision™ Microplate Pipetting System combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate compounds across a 96-well polypropylene plate, transfer compounds to 96- and 384-well cell plates and media from the cell plates to luminometer plates, as well as dispense Luciferin Detection Reagent to all luminometer plates.

BioTek Hybrid Detection

The Synergy™ H4 Hybrid Multi-Mode Microplate Reader combines a filter based and monochromator-based detection system in the same unit. The filter based system was used to read the luminescent signal from the P450-Glo™ and CellTiter-Glo® assays, and the fluorescent signal from the Resorufin Ethyl Ether assay using a 530/20 nm excitation filter, 590/35 nm emission filter, and 550 nm cutoff dichroic mirror.
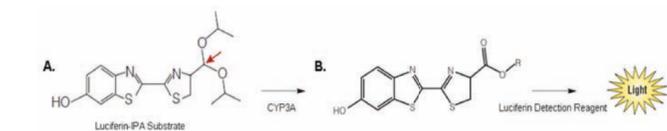
CYP1A/-3A Triplex Assay

Figure 1 – A. Luciferin-IPA substrate, 3 µM, is added to hepatocytes following the 48 hour compound incubation period. The cell-permeable substrate enters the cells. Activation of the PXR receptor causes an increase in CYP3A enzyme expression. CYP3A oxidizes the luminogenic substrate to create luciferin. Luciferin is also cell-permeable, and can move out of the cells into the media.

B. Upon addition of P450-Glo Luciferin Detection Reagent, the luciferin combines with luciferase and ATP to create light that is proportional to CYP3A activity.
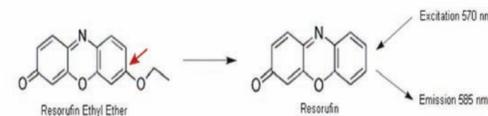

Figure 2 – Resorufin ethyl ether (7-ER), 2 µM, is added to hepatocytes following the 48 hour compound incubation period. The cell-permeable substrate enters the cells. Activation of the AHR receptor causes an increase in CYP1A enzyme expression. CYP1A cleaves the substrate to create the fluorescent substrate resorufin. Upon excitation at 570 nm, a fluorescent signal is then given off at 585 nm, which is proportional to CYP1A activity.
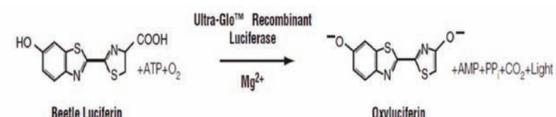

Figure 3 – The CellTiter-Glo cell viability reagent is added to hepatocytes following the resorufin fluorescent plate read. ATP from viable cells combines with luciferase and luciferin from the detection reagent to create a luminescent signal that is proportional to the number of viable cells in the well.
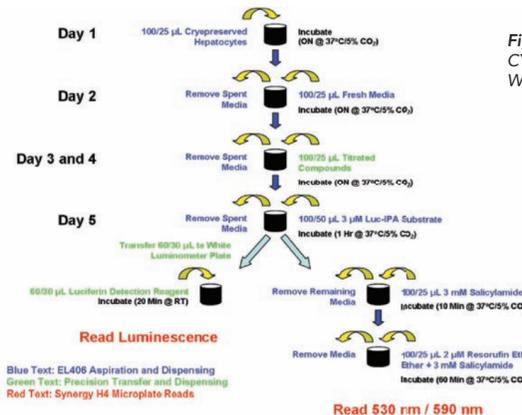
CYP1A/-3A Triplex Assay (Continued)

Figure 4 – 96- and 384-Well CYP1A/3A Triplex Assay Automated Workflow

Cells

Hepatocytes are the gold standard for induction studies because hepatocytes, the primary cell of the liver, have the full complement of enzymes, nuclear factors and co-factors at physiological levels that offer the best correlation to in vivo activity compared to cell lines and reporter gene systems. Plateable cryopreserved hepatocytes from Celsis In Vitro Technologies, were used with this project, due to the convenience, reproducibility and flexibility they offer, which is amendable to screening protocols.

Triplex Assay Validation

Hepatocyte Plating

The ability of the EL406 to accurately dispense hepatocytes to 96- and 384-well plates was verified by analyzing the %CV of the CellTiter-Glo values across all wells to contain cells in the assay (96-well: Columns 1-11; 384-well: Columns 1-22). 50,000 cells/well, and 10,000 cells/well were dispensed in 96- and 384-well formats, respectively. %CV values were 4.37% for 96-well dispensing, and 5.68% for 384-well dispensing.
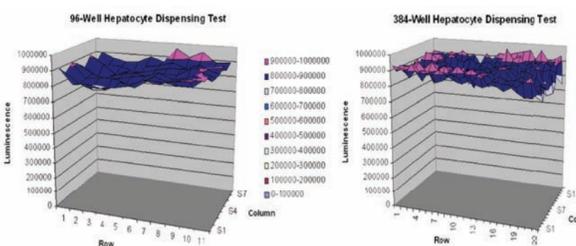

Figure 5 – Plating efficiency of hepatocytes by EL406

Z'-Factor Validation

Z'-Factor assays were performed to validate the CYP1A and -3A assays. Omeprazole was used as the control inducer for the CYP1A assay, while Rifampicin was used as the control inducer for the CYP3A assay. Forty-eight replicates of 10 µM or 0 µM compound were used as the positive and negative control, respectively.
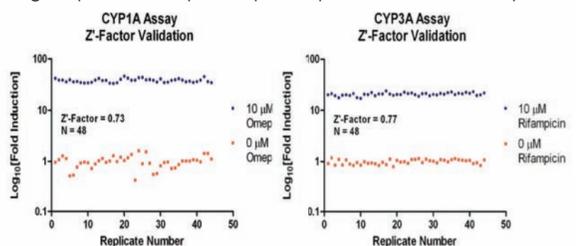

Figure 6 – Z'-Factor Validation Data

Triplex Assay Validation (Continued)

Fold Induction Calculation

P450-Glo and Resorufin fold induction were computed as follows. The average of the no cell background was subtracted from the individual signals for that compound concentration. This value was then divided by the CellTiter-Glo (CTG) 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 signals, divided by the basal CTG 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-Glo % cell viability was computed by dividing the CTG signal for each compound concentration by the CTG basal signal.

Compound EC_{50} Analysis

EC_{50} values for the control compounds Omeprazole and Rifampicin were computed to further validate the assay. Three runs from a single hepatocyte donor were performed to ensure data consistency. The assay was also performed in 96-well format as an additional control for the 384-well data. The EC_{50} values for each run fell within the average +/- ½ log concentration range of 2-21 µM for Omeprazole and 0.5-5.0 µM for Rifampicin.

The assay was also run using hepatocytes from other cell lots to analyze data consistency across multiple donors. The results for each test were once again within the concentration range determined from the original data set.
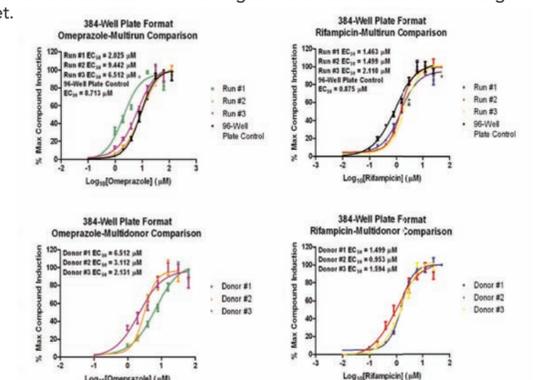

Figure 7 – Omeprazole and Rifampicin multi run and multi donor EC_{50} curves

Compound Induction/Inhibition Analysis

Eight compounds were tested with the triplex assay in dose response mode, including the known CYP1A inducers Omeprazole, β-Napthoflavone, 3-Methylcholanthrene, and Lansoprazole, and the known CYP3A inducers Phenylbutazone, Rifampicin, SR 12813, and Dexamethasone, CYP1A and -3A enzyme activity, and cell viability were computed for each compound. Resorufin and P450-Glo fold induction over basal activity was used to determine the effect of the compound on enzyme activity. Results were once again compared across multiple runs of the same hepatocyte donor, as well as across three separate hepatocyte donors.

CYP1A Fold Induction Values/Concentration Range (µM)				
	Omeprazole	β-Napthoflavone	3-Methylcholanthrene	Lansoprazole
Run #1	17-43 / 16-125	18-35 / 16-63	12-32 / 0.1-3	6-11 / 5-19
Run #2	14-26 / 16-125	15-31 / 16-63	7-25 / 0.1-3	7-13 / 5-19
Run #3	20-46 / 16-125	11-24 / 16-63	11-36 / 0.1-3	7-16 / 5-19
Donor #1	17-43 / 16-125	18-35 / 16-63	12-32 / 0.1-3	6-11 / 5-19
Donor #2	6-16 / 16-125	2-22 / 16-63	7-20 / 0.1-3	6-9 / 5-19
Donor #3	2-15 / 16-125	1-20 / 16-63	10-16 / 0.1-3	5-8 / 5-19
96-Well Control	9-14 / 16-125	8-16 / 16-63	4-17 / 0.1-3	4-8 / 5-19
FDA Guidelines Fold Induction Range	14-24	4-23	6-26	10
Concentration Range (µM)	25-100	33-50	1-2	10

Table 1 – Fold induction ranges for control compounds. Fold induction ranges given (left number range) for the concentration range listed (right number range). FDA guidelines[1] for fold induction and concentration range listed where applicable.

Compound Induction/Inhibition Analysis (Continued)

CYP3A Fold Induction Values/Concentration Range (µM)				
	Phenylbutazone	Rifampicin	SR 12813	Dexamethasone
Run #1	2-19 / 2-63	4-23 / 0.4-13	2-14 / 0.04-2.5	2-6 / 4-250
Run #2	3-19 / 2-63	4-28 / 0.4-13	3-22 / 0.04-2.5	3-10 / 4-250
Run #3	4-27 / 2-63	4-32 / 0.4-13	3-27 / 0.04-2.5	3-11 / 4-250
Donor #1	2-19 / 2-63	4-23 / 0.4-13	2-14 / 0.04-2.5	2-6 / 4-250
Donor #2	3-16 / 2-63	7-22 / 0.4-13	7-15 / 0.04-2.5	3-5 / 4-250
Donor #3	2-15 / 2-63	5-20 / 0.4-13	6-15 / 0.04-2.5	3-6 / 4-250
96-Well Control	1-5 / 2-63	4-11 / 0.4-13	1-8 / 0.04-2.5	2-4 / 4-250
FDA Guidelines Fold Induction Range		4-31		2.9-6.9
Concentration Range (µM)		10-50		33-250

Table 1 (Continued) – Fold induction ranges for control compounds. Fold induction ranges given (left number range) for the concentration range listed (right number range). FDA guidelines[1] for fold induction and concentration range listed where applicable.
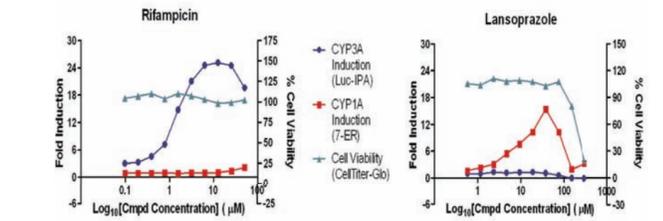

Figure 8 – Omeprazole and Lansoprozole fold induction/cell viability graphs

Fold induction and cell viability graphs were also generated to visualize the affect that each compound had on each assay across the entire concentration range tested. The graph for Rifampicin represents a typical graph generated from each data set. The Lansoprazole graph demonstrates how the cell viability measurement can be used to help explain the changes in fold induction that are seen across each compound titration. Lansoprazole induces CYP1A at lower concentrations of the compound, but then this effect decreases dramatically above 50 µM. By including the viability measurement, it becomes obvious that the decrease is due to cytotoxicity from the compound at higher concentrations.

Conclusions

1. The instrumentation used to perform the triplex assay provides an easy to use, dependable solution for dispensing cells, titrating compounds, delivering reagents, and reading the signal from assay plates.

2. The triplex assay, incorporating Promega's P450-Glo, and CellTiter-Glo reagents, as well as Resorufin Ethyl Ether, provides a straightforward way to assess the affects a compound may have on CYP1A and -3A activity.

3. Cryoplateable hepatocytes offer a reproducible and convenient alternative to freshly isolated hepatocytes and are amenable to HTS formats.

4. The combination of instrumentation, assay chemistry, and hepatocytes create an ideal solution to help fully understand the stimulatory and cytotoxic effects of a drug.

REFERENCES: U.S. Department of Health and Huan Services, FDA: Guidance for Industry Drug Interaction Studies Sept. 2006.