# A Simple, Robust Automated Multiplexed Cell-Based Assay Process for the Assessment of Mitochondrial Dysfunction and Cytotoxicity

**Brad Larson[1], Peter Banks[1], Tracy Worzella[2], Andrew Niles[2], and Timothy Moeller[3]**

[1]BioTek Instruments, Inc., Winooski, Vermont, USA • [2]Promega Corporation, Madison, Wisconsin, USA
[3]Celsis In Vitro Technologies, Baltimore, Maryland, USA

## Overview

The primary function of mitochondria is to generate >90% of a cell's energy in the form of ATP, through the process of oxidative phosphorylation. The impairment of this function can lead to negative effects on tissues such as reduced cellular function or cellular death. Recent studies have shown that an increasing number of drugs no longer on the market have negative effects on mitochondrial function in key organs such as the liver and heart. Therefore it is increasingly important to monitor the effects of lead compounds on mitochondrial function in relevant cell systems. The ability to incorporate a simple, rapid, multiplexed, predictive assay can make the detection of potential toxic effects easier to perform early on in the drug discovery process.

Here we demonstrate the automation and validation of such an assay in a high-density well format, using HepG2 and primary hepatocyte cell models.

## Introduction

Mitochondrial perturbation is a common mechanism of drug-induced toxicity. Recent advances in mitochondrial study have revealed that numerous drugs that were withdrawn from the market, or received Black Box warnings, demonstrate strong mitochondrial impairment in the liver or the heart. These include troglitazone (Rezulin), cerivastatin (Baycol), and nefazodone (Serzone). Therefore it is becoming increasingly important to focus on earlier identification of lead compounds that impact mitochondrial function during the discovery phase of drug development.

Here we demonstrate the utility of a multiplexed assay to assess cell membrane integrity changes (cytotoxicity), as well as mitochondrial function (ATP levels). Cytotoxicity is first assessed by measuring a distinct protease activity associated with necrosis using a fluorogenic peptide substrate (bis-AAF-R110) to measure "dead cell protease activity". The substrate cannot cross the intact membrane of live cells and therefore gives no signal with viable cells. Mitochondrial function is then measured by adding an ATP detection reagent, resulting in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The two assay readouts used together can distinguish between compounds that exhibit mitochondrial toxicity versus overt cytotoxicity. Mitochondrial toxicity will result in a decrease in ATP production with little to no change in membrane integrity. A cytotoxic effect, such as primary necrosis, will also show a decrease in ATP but in conjunction with loss of membrane integrity. In addition, the multiplexed nature of the assay decreases data variability that could be seen when running these two assays sequentially.

The entire process was automated, including dispensing cells, compound titration and transfer, and reagent additions. Primary hepatocytes, as well as the HepG2 cell line were used in a suspension format. The two cell models were compared in order to determine whether differences existed in the results seen from a cancer cell line and primary cells. Automated assay validation, effect of glucose-containing and non-glucose media (Crabtree effect), and pharmacology experiments were performed. Results demonstrate the utility of this automated, multiplexed assay to rapidly profile compounds for effects on mitochondrial function.

## BioTek Instrumentation

### BioTek Liquid Handling

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 dispense cells, serially titrate compounds across a 96-well PP plate, transfer compounds to the 384-well cell plates, as well as for reagent dispensing.

### BioTek 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 ATP Detection Assay, and the fluorescent signal from the Cytotoxicity Assay using a 485/20 nm excitation filter, 528/20 nm emission filter, and 510 nm cutoff dichroic mirror.

## Mitochondrial ToxGlo™ Assay

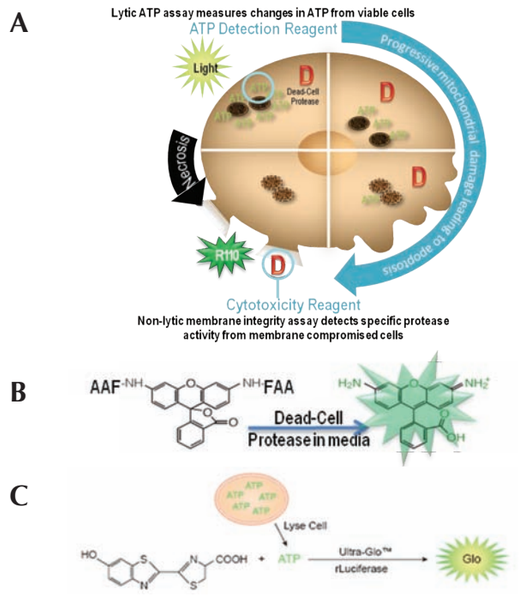


***Figure 1 – A. C****ell-Based, multiplexed method measures ATP (a proximal measure of mitochondrial function) in conjunction with a membrane integrity biomarker (protease) to distinguish primary mitochondrial dysfunction from secondary cytotoxic events directly in the same sample well.*

***B.*** *5X Cytotoxicity Reagent is added to the cells following the appropriate incubation time. The dead-cell protease substrate, which is unable to cross the intact membrane of live cells, is cleaved by a protease released from membrane compromised cells. Increased fluorescence correlates with dead cells.*

***C.*** *ATP Detection Reagent is then added to the wells following completion of the fluorescence detection step. The reagent lyses viable cells releasing ATP and generating a luminescent signal proportional to the amount of ATP present.*

## Suspension Hepatocytes

Hepatocytes are the most abundant cells of the liver and are involved in many critical functions of the body, including the majority of metabolism of endogenous and exogenous substances. This exposure to substances and their metabolites increases the susceptibility of the liver to cytotoxicity. Though cells lines derived from the liver, like HepG2 and HepaRG, are available, they lack the full complement of enzymes and transporters at physiologically relevant expression levels. Hepatocytes *in vitro* retain most of their *in vivo* function, especially phase I and phase II metabolism and transport activities and at physiologically relevant levels. Due to these attributes, hepatocytes are recognized as the gold standard for determining drug metabolism safety profiling and hepatotoxicity by researchers, pharmaceutical industry and regulatory agencies.

## Data Analysis

Luminescent or fluorescent values from wells containing media, treatments, and assay reagents were subtracted from raw values detected from cell containing wells. % Unstimulated Control was then computed using the following formula:

% Unstimulated Control = (Value(T) / Avg Value(U))*100

Where Value(T) equals the background subtracted value from wells containing compound, and Avg Value(U) equals the average value from background subtracted basal wells containing no compound.

## Cell and Compound Preparation

HepG2 cells were propagated in a medium formulation consisting High Glucose DMEM (Invitrogen Catalog #11995), 10% FBS and 1% Pen/Strep. After removal from the growth flask, the cells were resuspended in a glucose-free medium formulation consisting of No Glucose DMEM (Invitrogen Catalog #11966), 5 mM HEPES, 10 mM Galactose, 2 mM Glutamine, 1 mM Na-Pyruvate, and 1% Pen/Strep.

LiverPool™ cryopreserved human suspension hepatocytes (Celsis IVT Catalog #X008052) were thawed and resuspended in *InVitro*Gro HT Medium (Celsis IVT Catalog #Z99019). After the cells were spun down they were then resuspended in the glucose-free medium described above.

Compounds were also diluted from the 100% DMSO stocks in non-serum/non-glucose medium to the final 2X concentration before addition to the assay plates.

## Automated Assay Procedure

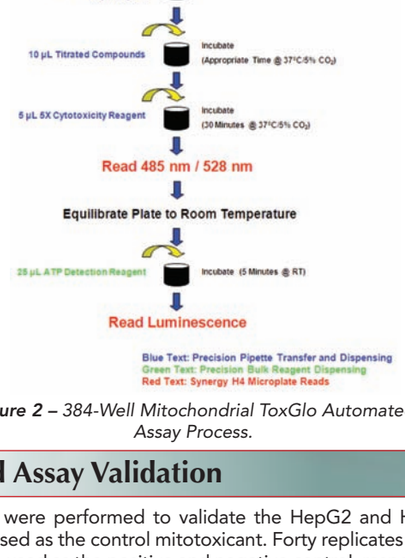


***Figure 2 –*** *384-Well Mitochondrial ToxGlo Automated Assay Process.*

## Automated Assay Validation

Z'-factor assays were performed to validate the HepG2 and Hepatocyte assays. Antimycin was used as the control mitotoxicant. Forty replicates of 100 µM or 0 µM compound were used as the positive and negative control, respectively. A two hour incubation time was used for compounds with each cell type.

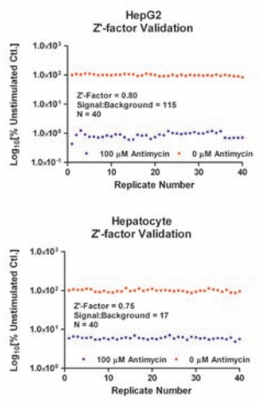


***Figure 3 –*** *Z'-factor validation data. Results from ATP Detection Assay shown here. Z' values ≥ 0.5 are indicative of an excellent assay according to Zhang et al., 1999. The difference in the signal:background seen between the HepG2 and hepatocyte cell models is due to the inability of the antimycin to totally kill hepatocytes, which leads to a higher signal from the negative control.*

## Cell Model Effect on ATP Production (Crabtree Effect)

Studies, including those described by Marroquin *et al.*, 2007, have shown that differences exist between cancer cell models and normal primary cells in how ATP is derived within the cell. Primary cells rely on mitochondrial oxidative phosphorylation to generate ATP. Cancer cells, in contrast, rely instead upon glycolysis when grown using typical high glucose medias. Only when glucose is substituted with galactose, will they revert back to using oxidative phosphorylation to generate ATP. This phenomenon, known as the Crabtree Effect, can cause compounds that would normally induce mitochondrial toxicity in an *in vivo* setting to appear as having no toxicological effect when tested using a cancer cell line in combination with high glucose medium.




***Figure 4 –*** *ATP production in cancer and normal primary cell models.*

The ability to detect this effect was tested here using the HepG2 cancer cell line and primary hepatocytes. Each cell type was resuspended and plated in either high or non-glucose medium, and then exposed to varying concentrations of the known mitotoxicant, antimycin, for two hours.

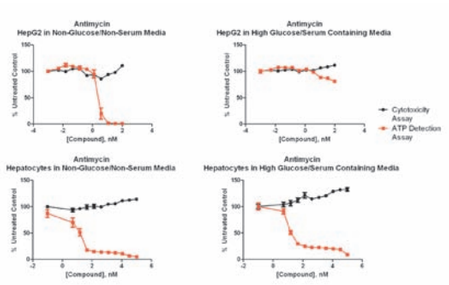


***Figure 5 –*** *Results confirm that the ability to detect toxic effects from compounds is obscured when using HepG2 cells plated in high glucose medium. A more in vivo-like response can be seen with HepG2 cells plated in non-glucose medium, or when using primary hepatocytes grown in either media.*

## Mitotoxicant Analysis

The ability of the automated 384-well assay to detect known mitotoxicants was further analyzed. Three known toxicants, antimycin, CCCP, and tamoxifen were tested using HepG2 and primary hepatocyte models. A known inducer of cellular necrosis, digitonin, was included as a cytotoxicity control. Staurosporine, a long-term apoptosis inducer was included as a negative control. The compounds were tested using cells resuspended and dispensed in non-serum/non-glucose medium. A two hour incubation period of compound with cells before addition of the detection reagents was incorporated for this test.

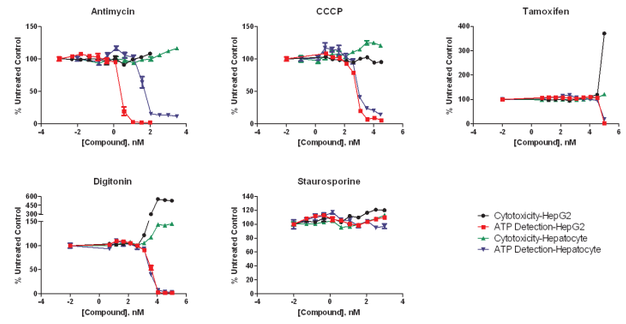


***Figure 6 –*** *A decrease in cellular ATP concentration was seen with increased concentrations of each of the three mitotoxicants tested with a two hour incubation. This is consistent with previously published literature references for antimycin (Tzung et al., 2001), CCCP (McCarron et al., 1999), and tamoxifen (Dykens et al., 2007). Tamoxifen demonstrated a decrease in cellular ATP only at the highest concentration tested, which may be indicative that the incubation time used here is not sufficient to see the complete effect of the compound. Digitonin also demonstrates an increase in signal from the cytotoxicity assay, in cooperation with a decrease in ATP concentration, indicative of it's necrosis inducing characteristics. Finally, staurosporine does not cause any change in signal with either assay, which also agrees with the known effects of the compound using this particular incubation time.*

## Variable Compound Incubation Time Analysis

The automated assay was further tested using compound-cell incubation times from one to six hours. This was completed to demonstrate the ability to use the assay, as well as cells in a suspension form, with extended incubation times up to six hours. A longer incubation time can allow for a more complete set of data to be generated for compounds that do not exhibit rapid toxic effects, or are less potent at lower concentrations.

Cells were dispensed as previously described. Compounds were then added to the cells at the appropriate time to create the proper incubation period.

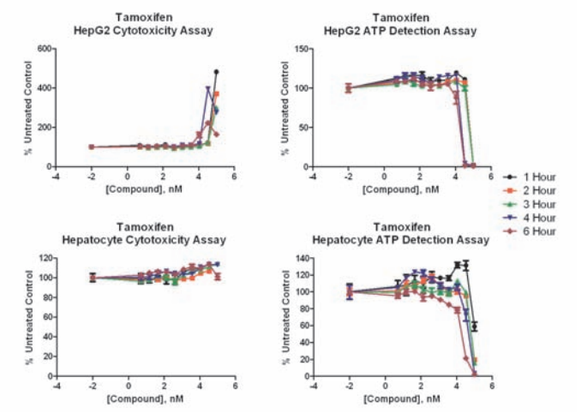


***Figure 7 –*** *The data shown for tamoxifen illustrates how results can vary using variable incubation times, and thus the need to test multiple exposures of compound with cells. The compound becomes more potent with increased exposure to both cell models. Changes in the signal from the cytotoxicity assay were also seen using HepG2 cells. This phenomenon, which is not seen in hepatocytes, may indicate a higher susceptibility to cytotoxicity from tamoxifen in the cancer cell model.*

## Cell Model Analysis

As previous experiments demonstrating the Crabtree Effect have shown, forcing cancer cell lines to generate ATP by mitochondrial oxidative phosphorylation can lead to a more *in vivo*-like response. However this change may still not yield the same results that would be generated by using a true primary cell model. This was tested by comparing the results from HepG2 cells and hepatocytes treated with the various mitotoxicants. All cells were once again resuspended and dispensed in non-serum/non-glucose medium to ensure that all cells are relying on mitochondrial generated ATP.

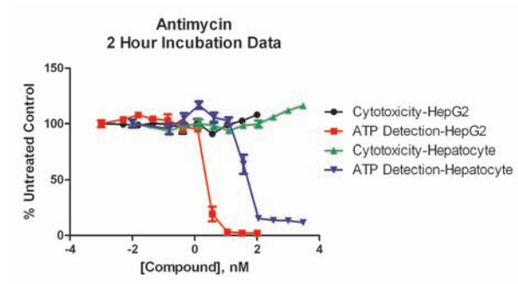


***Figure 8 –*** *The results for antimycin demonstrate that even when the HepG2 cells and hepatocytes are tested using the same media conditions, there are still differences in potency of the compound between the cell models. These results, as well as those in Figure 7, illustrate the need to use the most relevant cell model in mitochondrial toxicity testing.*

## Conclusions

1. The multiplexed assay provides an easy way to distinguish between compounds which negatively effect mitochondrial function, and those that effect the cell using other means such as primary necrosis.
2. The automated 384-well assay procedure, incorporating suspension cells, yields an efficient, yet robust way to perform the Mitochondrial ToxGlo™ assay in a high-throughput format.
3. Cryopreserved suspension hepatocytes offer a reproducible and convenient cell model, which generates the most *in vivo*-like results.
4. The combination of instrumentation, assay chemistry, and hepatocytes create an ideal solution to help make accurate predictions about the potential mitochondrial toxicity liabilities of lead compounds.