Simplifying Mechanistic Toxicity Testing Workflow with Automation

ABSTRACT

To demonstrate the use of automation for mechanistic toxicity testing, we present a workflow solution for cell-based assay research by combining a Hudson SOLO™ Multi-Channel Pipettor with Promega assay reagents and the GloMax®-Multi+ Detection System for luminescence and fluorescence detection in 96- and 384-well formats. The Mitochondrial ToxGlo™ Assay (Cat.# G8000, G8001) and ApoTox-Glo™ Triplex Assay (Cat.# G6320, G6321) were chosen to evaluate mechanistic toxicity produced by short- and long-term compound treatments of K562 cells. The model compound, CCCP, showed expected mitochondrial toxicity at early time points and subsequent induction of apoptosis and secondary necrosis with extended exposure.

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Introduction

In vitro cell-based assays have become an integral part of drug discovery, from target identification and validation through high-throughput screening. Automation is necessary for high-throughput screening but also can provide tangible benefits in characterizing lead compounds and guiding medicinal chemistry efforts where throughput is not typically a primary concern. For instance, studying the methods by which cells die aids in the development of targeted therapeutics for diseases such as cancer, as well as the evaluation off-target cytotoxic effects due to treatment. Small liquid handlers can be used for repetitive tasks including serially diluting compounds, dispensing cells and adding reagents. Automation of these basic tasks reduces hands-on setup time and helps to ensure assay consistency between days and different users.

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To demonstrate the advantages of a automated workflow, we programmed a small benchtop liquid handler from Hudson Robotics to perform basic cell-based assay setup tasks examining mechanistic toxicity. The Promega Mitochondrial ToxGlo™ and ApoTox-Glo™ Triplex Assays and the GloMax®-Multi+ Detection System (Cat.# E9032) were used to complete the workflow (Figure 1). We used K562, a chronic myelogenous leukemia cell line, to test specific toxicity events due to treatment with CCCP (carbonyl cyanide m-chlorophenylhydrazone), a mitochondrial uncoupling agent. The Mitochondrial ToxGlo™ Assay, was chosen to assess effects of CCCP on mitochondrial function in a short-term treatment (2 hours) in galactose-substituted medium. This study was conducted in galactose and glucose containing medium because it is now well-documented that highly proliferative cancer cell lines grown soley in glucose-containing medium typically utilize glycolysis for ATP production and as a means of meeting biosynthetic precursor requirements (the Crabtree Effect). Therefore by replacing glucose with galactose in the assay medium the bioenergetic balance is shifted to favor oxidative phosphorylation for ATP generation, making the cells more susceptible to the effects of a mitochondrial toxicant (1) . The ApoTox-Glo™ Assay was employed to address the mechanistic basis of a longer term CCCP exposure.

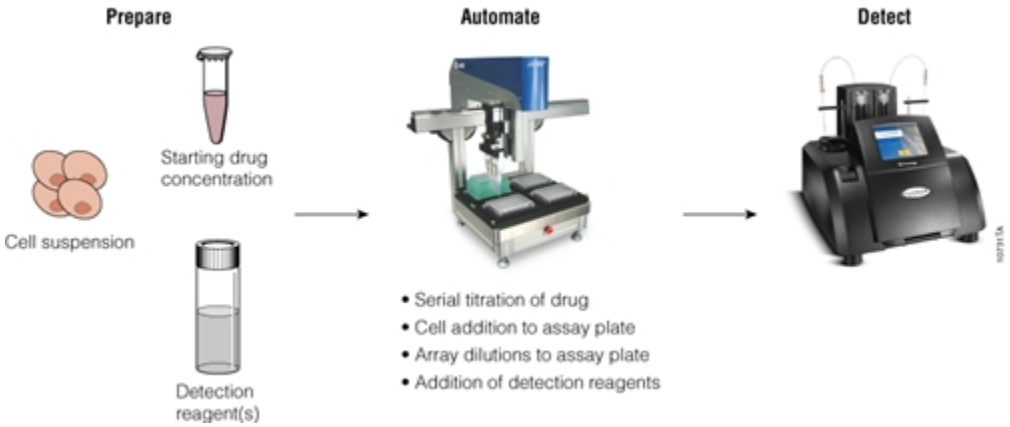

Figure 1. A common workflow is followed for cell-based assay experiments, regardless of the assay being performed.

The Mitochondrial ToxGlo™ Assay (Figure 2, Panel A) is a multiplexed, sequential-addition assay that measures biomarkers associated with cell membrane integrity and ATP levels. The membrane integrity marker serves as a control for distinguishing between primary mitochondrial toxicity and primary necrosis, as both mechanisms result in a decrease in ATP. Cell membrane integrity is first assessed with a nonlytic buffer containing a fluorogenic peptide substrate (bis-AAF-R110) that measures the activity of a dead-cell protease released into the medium when the cell membrane becomes compromised. The dead cell substrate cannot cross the intact membrane of live cells and therefore produces minimal signal from viable cells. Fluorescent signal (485nmEx, 520nmEM) is proportional to the number of dead cells in the well. The ATP Detection Reagent is then added to lyse the cells, releasing ATP and generating a luminescent signal proportional to the amount of ATP present (2) .

The ApoTox-Glo™ Triplex Assay (Figure 2, Panel B) is a multiplexed, sequential addition assay that measures biomarkers associated with cell viability, cytotoxicity and apoptosis. Cell viability and cytotoxicity are measured first with the addition of two substrates that detect viable and membrane-compromised cells (GF-AFC, 400nmEx/505nmEM and bis-AAF-R110, 485nmEx/520nmEM). The GF-AFC substrate is cell permeant and measures a protease associated with live cells. The live-cell protease becomes inactivated once it is released into the medium and therefore produces minimal signal from dead cells. The dead-cell protease substrate works as described previously for the Mitochondrial ToxGlo™ Assay (3) . The Caspase-Glo®-3/7 Reagent is then added to measure caspase-3/7 activity, a hallmark of apoptosis. Caspase-3/7 cleaves a proluminogenic DEVD-luciferin substrate, releasing luciferin and generating light in a firefly luciferase reaction. Light output is proportional to caspase-3/7 activity (apoptosis) (4) .

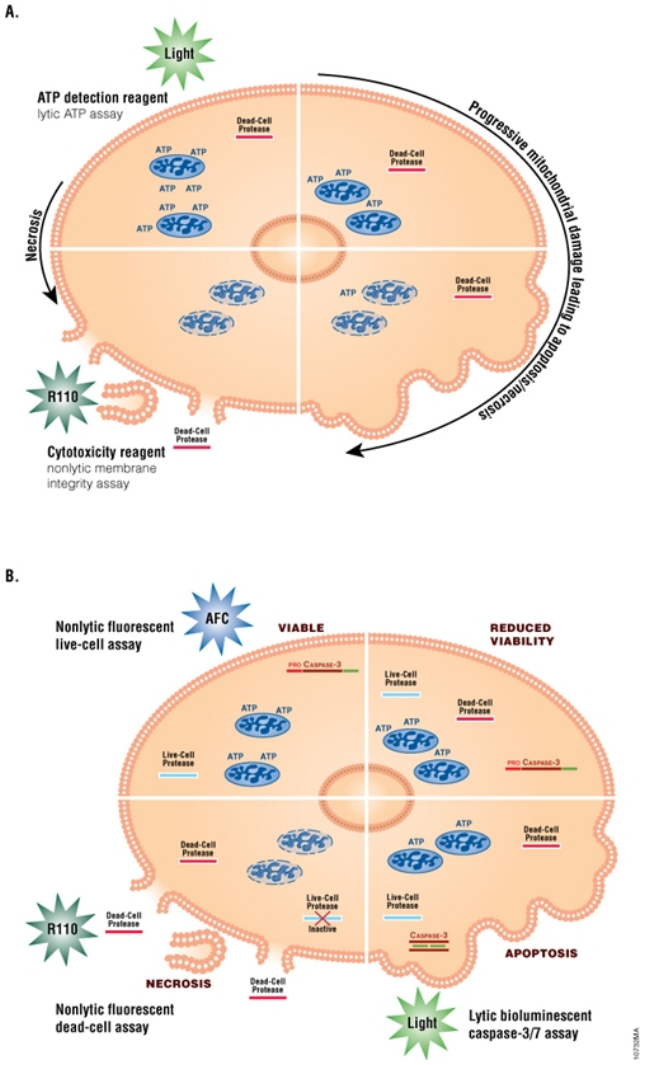

Figure 2. Mitochondrial ToxGlo™ and ApoTox-Glo™ Triplex Assay Schematics. The Mitochondrial ToxGlo™ Assay (Panel A) is a multiplexed, sequential-addition assay that measures biomarkers associated with cell membrane integrity and ATP levels. Cell membrane integrity is first assessed with a fluorogenic peptide substrate (bis-AAF-R110) that measures the activity of a dead-cell protease released into the medium when the cell membrane is compromised. Fluorescent signal is proportional to the number of dead cells in the well. Next, the ATP Detection Reagent is added to lyse the cells and release ATP. This generates a luminescent signal proportional to the amount of ATP present. The ApoTox-Glo™ Triplex Assay (Panel B) is a multiplexed, sequential addition assay that measures biomarkers associated with cell viability, cytotoxicity and apoptosis. Cell viability and cytotoxicity are measured first by adding two substrates that

detect viable and membrane-compromised cells (GF-AFC and bis-AAF-R110). GF-AFC is cell permeant and measures a protease associated with live cells. The live-cell protease becomes inactivated once it is released into the medium and produces only a minimal signal from dead cells. The bis-AAF-R110 measures a dead-cell protease.

Methods

Starting concentrations of CCCP, reagents and cell suspensions were manually prepared and arrayed on the deck of the Hudson SOLO™ Multi-Channel Pipettor. The Hudson SOLO™ Instrument was used for all liquid handling steps required to set up the experiments conducted for this mechanistic toxicity study. Assay plates were manually transferred between the liquid handler and shaker, cell culture incubator and microplate reader (Figure 3). The following protocols highlight the processes for performing the assays described here.

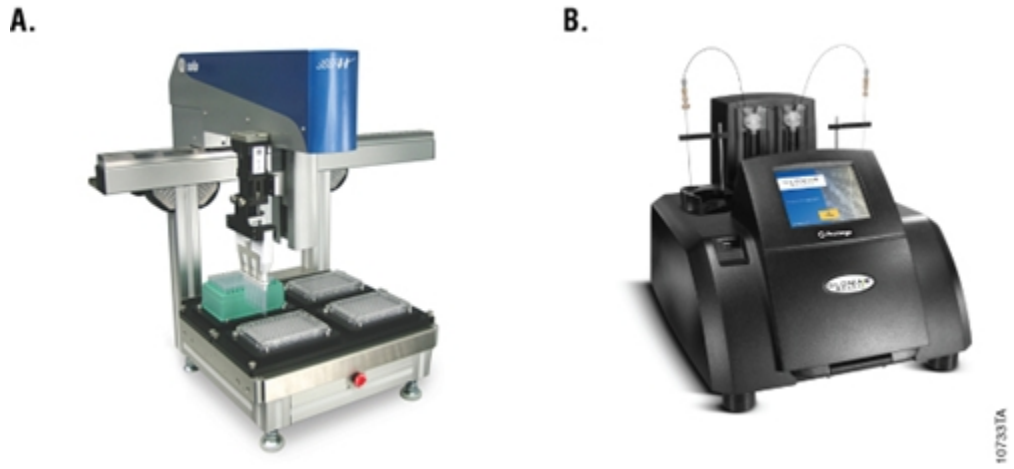

Figure 3. The Hudson SOLO™ Multi-Channel Pipettor and Promega GloMax®-Multi+ Detection System. Panel A. The Hudson SOLO™ includes an 8-channel 200µl pipettor, four deck positions, and SOLOSoft control software. Panel B. The GloMax®-Multi+ Detection System with Instinct™ software is a multifunctional plate reader capable of luminescence, fluorescence and absorbance detection.

MITOCHONDRIAL TOXGLO™ ASSAY (96-WELL FORMAT)

1. K562 cells were resuspended in serum-free, glucose-free RPMI 1640 medium supplemented with galactose or serum-free, glucose-containing RPMI 1640 medium to a density of 2×10^5 cells/ml.
2. Each cell suspension was dispensed in 50µl aliquots into a separate 96-well plate (Corning Cat.# 3917).
3. A 100µM working solution of CCCP was prepared in the respective assay medium and added to Column 1 of the dilution plate (Corning Cat.# 3370 plate).
4. One hundred microliters of each medium formulation was added to Columns 2–12 of the dilution plate.
5. Serial 1:2 dilutions of CCCP were performed across each dilution plate.
6. Each compound dilution was transferred to each assay plate in 50µl aliquots.
7. The plates were mixed on a 2mm orbital shaker for 1 minute and placed into a 37°C/5% CO_2 incubator for two hours.
8. Twenty microliters of the 5X Cytotoxicity Reagent was added to each well.
9. The plates were mixed on a 2mm orbital shaker for 1 minute and placed into a 37°C/5% CO_2 incubator for 30 minutes.
10. Fluorescence (485nm$_{Ex}$, 520nm$_{EM}$) was measured with the GloMax®-Multi+ Detection System.
11. The plates were cooled for 15 minutes at room temperature.
12. One hundred microliters of the ATP Detection Reagent was added to each well.

13. The plates were mixed on a 2mm orbital shaker for 1 minute and incubated at room temperature for 10 minutes.
14. Luminescence was measured with the GloMax®-Multi+ Detection System.

APOTOX-GLO™ ASSAY (384-WELL FORMAT)

1. K562 cells were resuspended in RPMI 1640 + 10% FBS to a density of 5×10^5 cells/ml.
2. The cell suspension was dispensed in 10µl aliquots into each well of a 384-well plate (Corning # 3570).
3. A 20µM working solution of CCCP was prepared in assay medium and added to Column 1 of the dilution plate (Corning Cat.# 3370 plate).
4. One hundred microliters of the assay medium was added to Columns 2–12 of the dilution plate.
5. Serial 1:2 dilutions of CCCP were performed across the dilution plate.
6. Ten-microliter aliquots of each compound dilution were transferred to the assay plate in replicates of four.
7. The plates were mixed on an electromagnetic shaker for 1 minute and placed into a 37°C/5% CO_2 incubator for 24 hours.
8. The 2X Viability/Cytotoxicity Reagent was added to each well in 20µl aliqouts.
9. The plates were mixed on an electromagnetic shaker for 1 minute and placed into a 37°C/5% CO_2 incubator for 30 minutes.
10. Fluorescence (485nm$_{Ex}$, 520nm$_{EM}$ and 405nm$_{Ex}$, Em505$_{EM}$) was measured with the GloMax®-Multi+ Detection System.
11. Forty microliters of the Caspase-Glo® 3/7 Reagent was added to each well.
12. The plates were mixed on an electromagnetic shaker for 1 minute and incubated at room temperature for 30 minutes.
13. Luminescence was measured with the GloMax®-Multi+ Detection System.

Results

There were dramatic differences in data generated by the Mitochondrial ToxGlo™ Assay between the cells assayed for two hours in galactose-containing medium and those assayed for two hours in glucose-containing medium (Figure 4, Panel A). This observation is consistent with the principle tenets of the Crabtree Effect because the cells utilizing galactose as an energy source relied primarily on oxidative phosphorylation (and hence mitochondrial function) to generate ATP. As a result, a potent and dose-dependent effect on ATP production was observed. The cells assayed in glucose-containing medium relied primarily on glycolysis to generate ATP so the mitochondrial toxicity effects of CCCP were not revealed by the ATP measurement. We noted minimal effects on membrane integrity in both culture conditions, suggesting that observed decreases in ATP were due to specific effects on mitochondria and not primary necrosis.

Twenty-four hour treatment with CCCP shows a toxicity profile consistent with apoptosis and secondary necrosis (Figure 4, Panel B). There was a dose-dependent increase in apoptosis with a corresponding increase in cytotoxicity. Cell viability also decreased as apoptotic cells progressed to secondary necrosis.

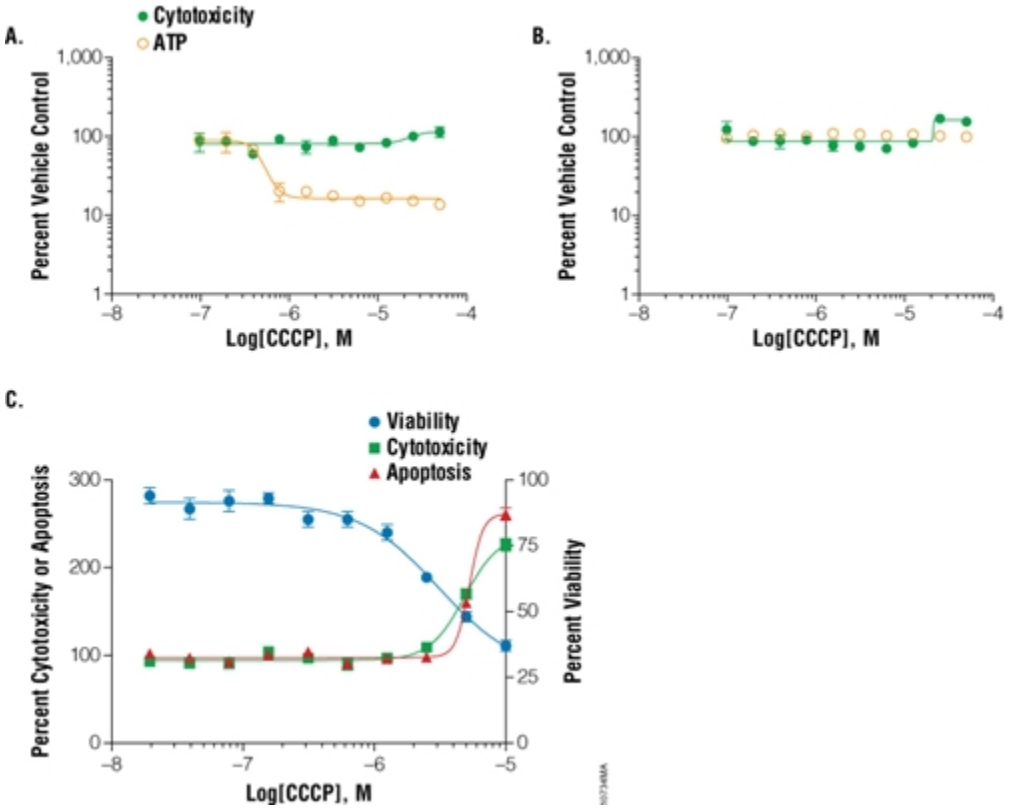

Figure 4. Results from a 24-hour incubation of K562 cells with CCCP using the Mitochondrial ToxGlo™ and ApoTox-Glo™ Triplex Assays. The Mitochondrial ToxGlo™ Assay uses a fluorogenic peptide substrate (bis-AAF-R110) that measures the activity of a dead-cell protease released into the medium when the cell membrane is compromised. Fluorescent signal is proportional to the number of dead cells in the well. An ATP Detection Reagent lyses the cells and release ATP. This generates a luminescent signal proportional to the amount of ATP present. The ApoTox-Glo™ Triplex Assay measures cell viability, cytotoxicity and apoptosis. Cell viability and cytotoxicity are measured first by adding GF-AFC and bis-AAF-R110. GF-AFC measures a protease associated with live cells and bis-AAF-R110 measures a dead-cell protease. Panel A. Mitochondrial ToxGlo™ Assay results with cells grown in Galactose Medium. Panel B. Mitochondrial ToxGlo™ Assay results with cells grown in Glucose Medium. Panel C. ApoTox-Glo™ Triplex Assay results. All assays were performed as described in the Methods.

Conclusions

We have demonstrated that small, personalized liquid handling and detection instruments offer significant utility for performing cell-based assays in a lower-throughput setting. Basic tasks required to perform a cell-based assay are the same regardless of the assay being performed. Programming these tasks into a liquid handler creates a system with endless possibilities for assay development or lead development as well as any other process that requires repetitive pipetting. We have also shown that both 96- and 384-well plate formats are amenable to automation on smaller platform pipetting and detection systems. This will provide flexibility if throughput needs increase.

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