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Abstract

Cell-based assays are useful tools to investigate *in vitro* responses to a variety of target compounds and cell-signaling molecules. Multiparametric measures are often desirable, particularly in secondary screening, to generate more biologically relevant data. The evaluation of multiple parameters can become quite labor intensive and time consuming if conducted in parallel, so the use of simple multiplex assays executed with automation help to improve efficiency and reduce operator involvement.

Here, we introduce an automated ApoTox-Glo cell-based assay application for simultaneously measuring three parameters: cell viability, cytotoxicity, and apoptosis. The method combines two fluorescent and luminescent assay chemistries offered by Promega (Caspase-Glo® 3/7 and MultiTox-Fluor™ Assays) in the same assay well to extract information about viability, cytotoxicity and caspase activation events. These parameters are particularly useful to define mechanisms associated with a cytotoxic profile. All dispensing steps are automated and performed using the BioTek Precision™ Microplate Pipetting System. Fluorescence and luminescence measurements are performed using the BioTek Synergy™ 2 Multi-Mode Microplate Reader.

Fluorescent and Luminescent ApoTox-Glo Assay

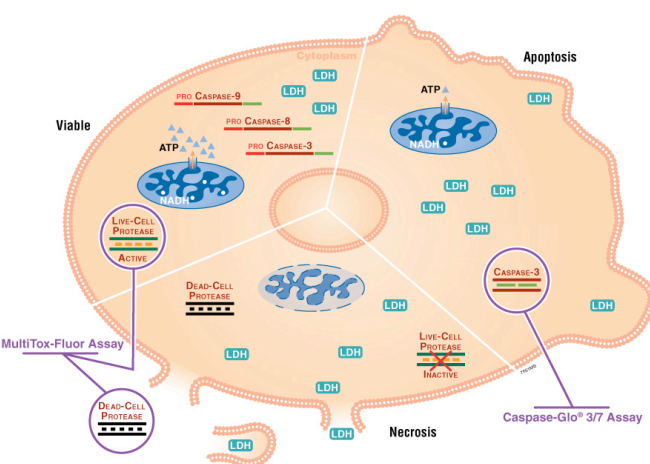


Figure 1. The ApoTox-Glo assay principle using the Promega MultiTox-Fluor™ Assay & Caspase-Glo® 3/7 Assays.

The ApoTox-Glo assay is comprised of the Promega MultiTox-Fluor™ and Caspase-Glo® 3/7 Assays. The MultiTox-Fluor™ Assay is a non-lytic chemistry that allows measurement of live and dead cells in a single sample well. Specifically, for live cell assessment, live-cell protease activity is measured by the fluorogenic, cell-permeant peptide substrate Gly-Phe-7-amino-4-trifluoromethyl coumarin (GF-AFC). This live-cell protease activity marker labels only live cells because it becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium, and thus does not contribute to the dead cell

measurement. For dead cell assessment, a second protease activity marker, the cell-impermeant peptide substrate bis-(Ala-Ala-Phe)-rhodamine 110 (bis-AAF-R110), is used to measure the activity of a dead-cell protease from cells that have lost membrane integrity and leaked the biomarker into the surrounding culture medium. The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase-3 and -7 activities in cultures of cells, which are indicative of apoptosis. The assay provides a proluminescent caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD. This substrate is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. The amount of light produced correlates with caspase-3/7 activity. Together, these assays provide a researcher with three pieces of data per well (cell viability, cytotoxicity, and caspase activity) which can be used to more accurately profile compound effects on cells.

Lab Automation Instrumentation

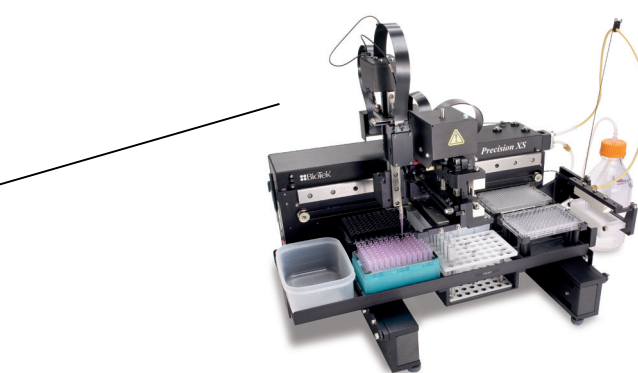
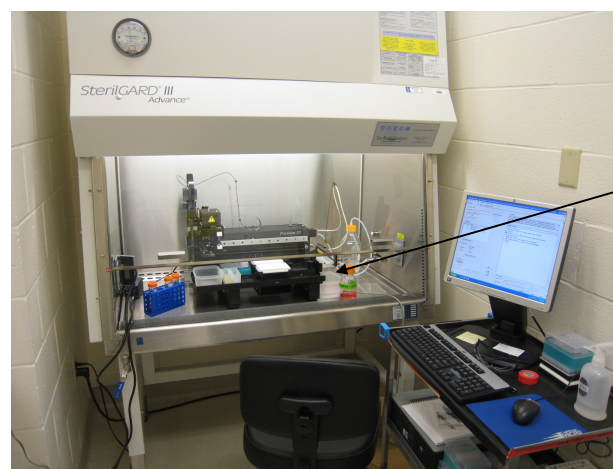


Figure 2. The BioTek Precision™ XS Automated Sample Processor is a precise and compact liquid handling system.

The BioTek Precision™ Microplate Pipetting System is a precise liquid handling system used for dispensing steps and is easily programmed to implement the ApoTox-Glo assay. This compact robotic instrument combines a single-channel sample processing head, an eight-channel pipetting head, and an eight-channel bulk reagent dispenser to control all liquid handling steps of the assay including the dilution of compounds, sterile dispensing and treatment of cells, and addition of assay reagents. The small footprint of the Precision™ enables sterile experimentation inside a biological safety cabinet, which is critical for any assay with treatment times beyond 12–24 hours



Figure 3. The BioTek Synergy™ 2 is a high-performance multi-mode microplate reader.

The BioTek Synergy™ 2 Multi-Mode Microplate Reader is used for measuring fluorescence at two wavelengths and luminescence in the Triplex assay. The fluorescence detection system uses deep blocking filters (400Ex/505Em for the live-cell assay; 485Ex/520Em for the dead-cell assay) and dichroic mirrors for excellent performance in research and screening applications, such as the Triplex assay.

ApoTox-Glo Assay Protocol

The Precision Power™ PC Software that accompanies the instrument makes stepping through the assay protocol very straightforward and easy. Specifically, a 5-step ApoTox-Glo assay protocol was created (described below).

1. Dispense cells in culture medium into assay plates.
2. Serially dilute and dispense compounds across the assay plate and incubate for the desired test exposure period (i.e., 24 hours).
3. Dispense MultiTox-Fluor™ Reagent to all wells of the assay plate, shake briefly, incubate at 37°C for 30 minutes, and measure fluorescence (live-cell fluor 400Ex/505Em; dead-cell fluor 485Ex/520Em).
4. Dispense Caspase-Glo® 3/7 Reagent to all wells of the assay plate, shake briefly to lyse cells, incubate at room temperature for 30 minutes, and measure luminescence.
5. Repeat steps 3-4 at each treatment time point.

ApoTox-Glo Assay Results

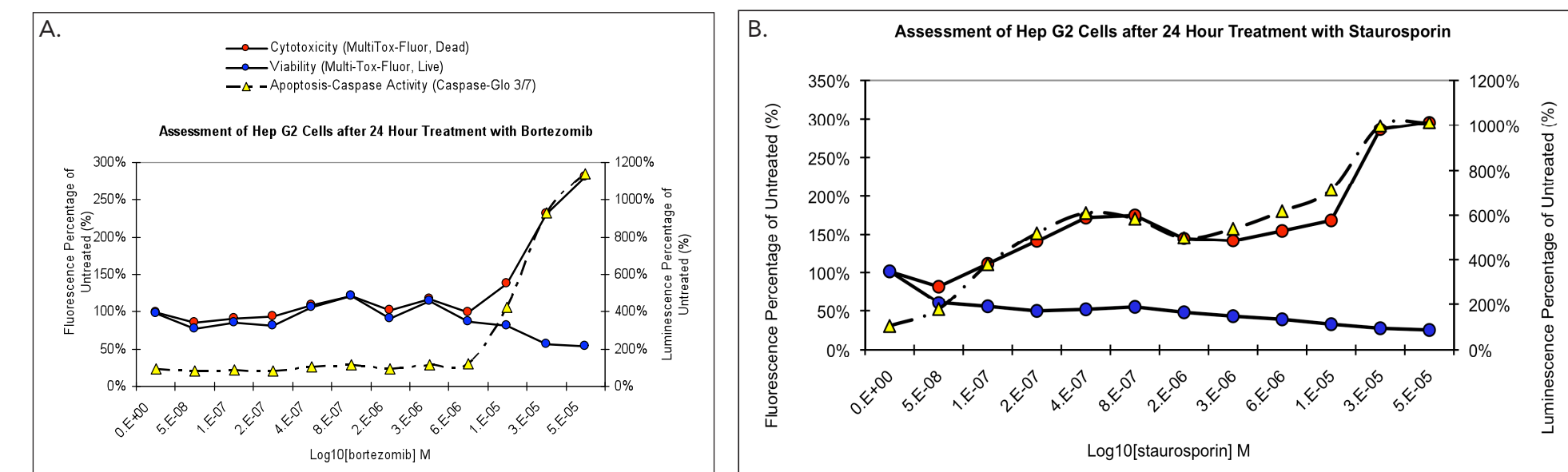


Figure 4. ApoTox-Glo assay assessment of Hep G2 cells after 24 hour treatment with bortezomib (A) or staurosporine (B).

Proteolytic biomarkers for cell viability and cytotoxicity were measured using the MultiTox-Fluor™ Assay. Caspase-3/7 activity was then measured in the same well using the Caspase-Glo® 3/7 Assay. As expected, both staurosporine and bortezomib compound treatments for 24 hours resulted in a dose-dependent decrease in viability, an increase in cytotoxicity, and an increase in caspase-3/7 activity consistent with apoptosis.

Hep G2 cells were plated in 384-well format at a density of 5,000 cells in 10μL volumes of DMEM plus 10% fetal bovine serum and allowed to equilibrate for two hours. Staurosporine and bortezomib were twofold serially diluted and added to wells in 10μL volumes. Plates were incubated at 37°C in 5% CO₂ for 24 hours. MultiTox-Fluor™ Reagent was prepared as 10μL of each substrate in 2.5mL Assay Buffer, and 5μL was used per well. The plate was mixed and incubated for 30 minutes at 37°C. Fluorescence measures of viability and cytotoxicity were read at two wavelengths on the BioTek Synergy™ 2 Multi-Mode Microplate Reader. Caspase-Glo® 3/7 Reagent was then added in an additional 25μL volume and luminescence measured on the BioTek Synergy™ 2 Multi-Mode Microplate Reader after 30 minutes at room temperature.

Summary

Together Promega cell-based assays can be used with the BioTek Precision™ Microplate Pipetting System to provide a simple automated Triplex application to evaluate the effects of compounds on cell viability, cytotoxicity, and apoptosis over time.

Implementing this automated ApoTox-Glo assay provides a small-scale, cost-effective cell-based screening method particularly well-suited for *in vitro* toxicology applications in academic labs, growing biotechnology companies, and for secondary screening environments.

Synergy™ 2 Multi-Mode Microplate Reader provides detection capability for all three assays.