Application Note

Automated ApoTox-Glo Assay to Assess Cell Viability, Cytotoxicity and Apoptosis

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Using the Precision™ Automated Pipetting System to perform serial dilution of compounds to test for cell toxicity, viability and manner of cell death (necrosis or apoptosis) using a multiplexed approach combining fluorescence and luminescence readouts suitable for detection with the Synergy™ 2 Multi-Mode Microplate Reader.

Introduction

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 helps 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™ XS Automated Sample Processor.

Fluorescent and Luminescent ApoTox-Glo Assay

The ApoTox-Glo assay described here 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.

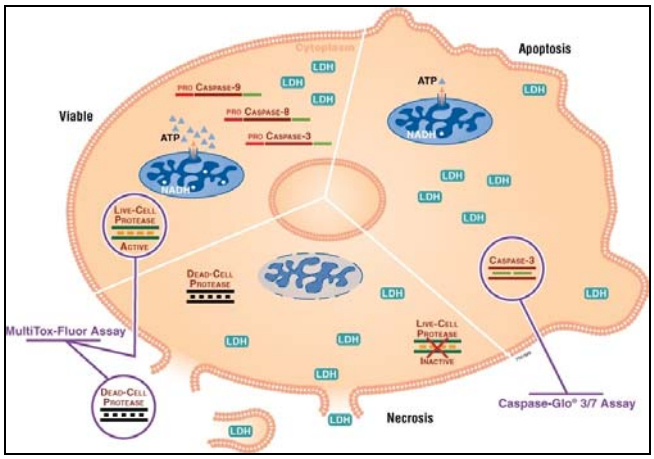


Figure 1. The ApoTox-Glo assay principle using the MultiTox-Fluor™ and Caspase-Glo® 3/7 Assays. The MultiTox-Fluor™ Multiplex Cytotoxicity Assay is a single-reagent-addition, homogeneous, fluorescent assay that measures the number of live and dead cells simultaneously in culture wells. The Caspase-Glo® 3/7 Assay provides a homogeneous luminescent assay that measures caspase-3/7 activities.

Following use of the MultiTox-Fluor™ Assay, a second assay is performed to obtain a third type of data from the cell population. 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 affects on cells. The ApoTox-Glo assay also has a built-in control for assay normalization to help correct for well-to-well and day-to-day variability within a cell-based system.

Automated Dispensing Instrumentation

The BioTek Precision™ XS Automated Sample Processor 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 XS enables sterile experimentation inside a biological safety cabinet, which is critical for any assay with treatment- times beyond 12–24 hours.

The BioTek Synergy™ 2 Multi-Mode Microplate Reader is used for measuring fluorescence at two wavelengths and luminescence in the ApoTox-Glo 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 ApoTox-Glo assay.

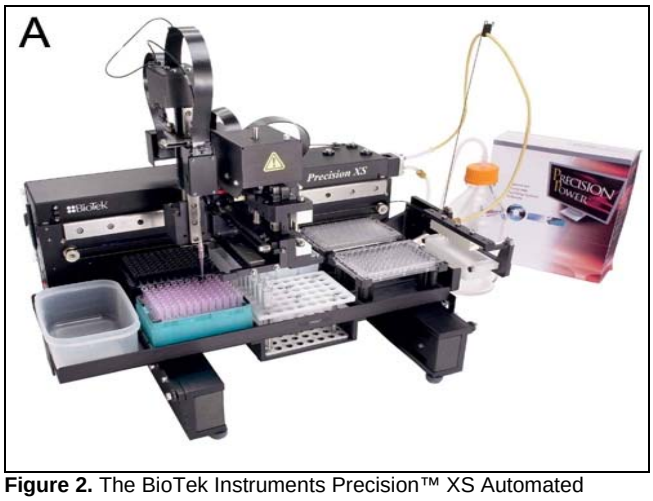


Figure 2. The BioTek Instruments Precision™ XS Automated Sample Processor and Precision Power Software.

ApoTox-Glo Assay Protocol

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. Saurosporine 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.

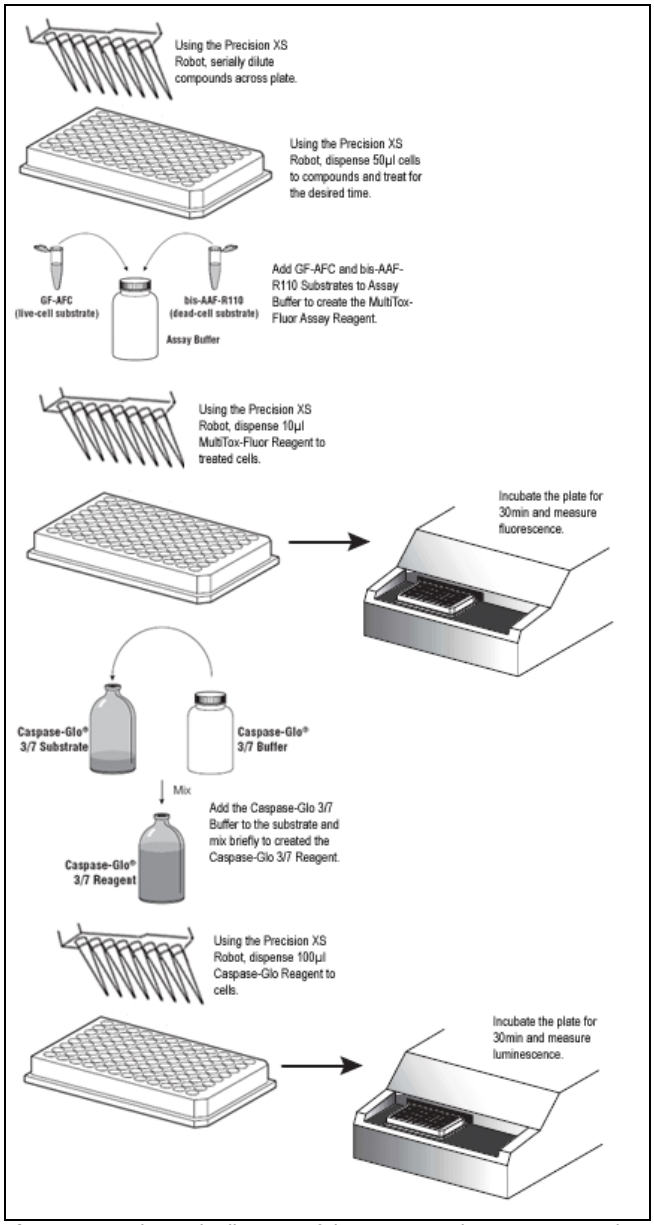


Figure 3. A schematic diagram of the ApoTox-Glo assay protocol. The MultiTox-Fluor™ Assay is performed first with downstream Caspase-Glo® 3/7 Assay.

The Precision Power™ PC Software that accompanies the instrument makes stepping through the assay protocol very straight-forward and easy. Specifically, a 5-step ApoTox-Glo assay protocol was created (described below and illustrated in Figure 3).

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., 4, 8, 12, 24, 48 hours).

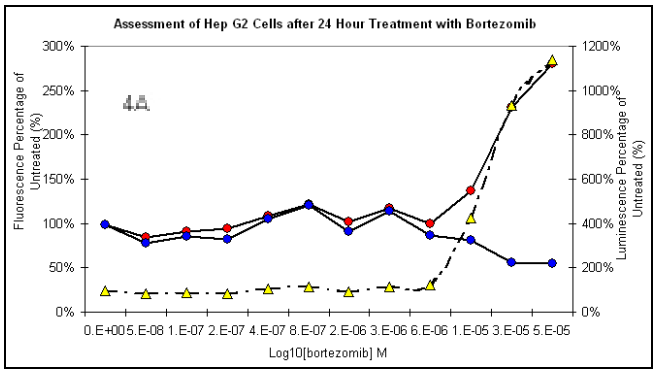
3. Dispense MultiTox-Fluor™ Reagent to all wells, shake briefly, incubate at 37°C for 30 minutes, and measure fluorescence (live-cell fluor 400Ex/505Em; dead-cell fluor 485Ex/520Em).

4. Dispense Caspase-Glo® 3/7 Reagent to all wells, 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

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 bortezomib (Figure 4) and staurosporine (Figure 5) 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.



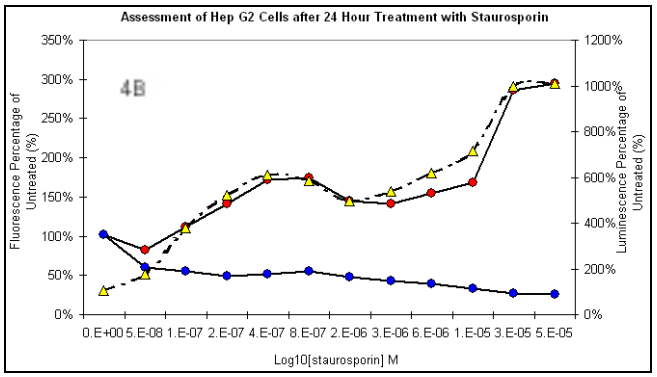


Figure 4. ApoTox-Glo assay results. A. Assessment of Hep G2 cells after 2 hour treatment with bortezomib. B. Assessment of Hep G2 cells after 4 hour treatment with staurosporine.

Summary

Together Promega cell-based assays can be used with the BioTek Precision™ XS Automated Sample Processor and Synergy 2 Multi-Mode Microplate reader to provide a simple automated multiplexed solution to evaluate simultaneously the effects of compounds on cell viability, cytotoxicity, and apoptosis. 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, small biotechnology companies, and for secondary screening environments.

General References

1. Abigail Farfan, Thomas Yeager, Rich Moravec, and Andrew Niles. Multiplexing Homogeneous Cell-Based Assays *Cell Notes* Issue 10 (2004) 15-18.

2. Andrew Niles, Richard Moravec, P. Eric Hesselberth, Michael Scurria, William Daily, and Terry Riss. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers *Analytical Biochemistry* Volume 366, Issue 2 (2007) 197-206.

3. Andrew Niles, Richard Moravec, and Terry Riss. Update on in vitro cytotoxicity assays for drug development *Expert Opin. Drug Discov.* Volume 3, Issue 6 (2008) 655-669.

4. TB348 Promega MultiTox-Fluor™ Multiplex Cytotoxicity Assay Technical Bulletin

5. TB323 Promega Caspase-Glo® 3/7 Assay Technical Bulletin

6. Tracy Worzella, Michael Busch, and Andrew Niles. High-Throughput Automation of Multiplexed Cell-based Assays for Viability and Cytotoxicity *Cell Notes* Issue 20 (2008) 26-29.

7. Halina Zakowicz, Trista Schagat, David Yoder, and Andrew Niles. Measuing Cell Health and Viability Sequentially by Same-Well Multiplexing Uisng the GloMax®-Multi Detection System *Promega Notes* Issue 99 (2008) 25-28.

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