Determining the Predictive Mechanism of Toxicity Using a Single-Well Multiplexed Assay

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ABSTRACT

The ApoTox-Glo[™] Triplex Assay is intended for measurement of three parameters: cell viability, cytotoxicity, and caspase activation events in a single well. The assay combines one luminescent and two fluorescent technologies into one kit. This combination allows the assay to provide information about cell health, toxicity and apoptosis events. These parameters taken together provide a broader picture of what is occurring inside cells and is particularly well suited for in vitro toxicology applications in academic labs, growing biotechnology companies, as well as in secondary screening environments.

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Introduction

Cell-based assays are useful tools to investigate in vitro responses to a variety of target compounds and cell-signaling molecules. Using compatible assays capable of measuring multiple parameters in a single well (i.e., multiplexing) with the same population of cells is often desirable, particularly in secondary screening, to generate more biologically relevant data. Multiplexing assays also helps to provide normalized controls, save materials and reduce the time- and labor-intensive tasks of performing each assay individually.

By combining three assays into one multiplex assay, the ApoTox-Glo[™] Triplex Assay provides a broader picture of what is occurring inside cells, specifically information about cell health, toxicity, and apoptosis events.

Here, we introduce a new multiplexing cell-based assay product called the ApoTox-Glo[™] Triplex Assay. This triplex assay is intended for same-well measurement of three parameters: cell viability, cytotoxicity, and caspase activation events. The chemistry combines one luminescent and two fluorescent technologies into one convenient kit for users. Each of the three subsets of the assay also are available individually (CellTiter-Fluor[™], CytoTox-Fluor[™], and Caspase-Glo[®] 3/7), allowing users to optimize individual parameters of the assay.

By combining three assays into one multiplex assay, the ApoTox-Glo[™] Triplex Assay provides a broader picture of what is occurring inside cells, specifically information about cell health, toxicity, and apoptosis events. Together these parameters help better define mechanisms associated with a cytotoxic profile. The assay is especially well suited for in vitro toxicology applications in academic labs, small biotechnology companies, and for secondary screening environments.

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How the Assay Chemistry Works

The ApoTox-Glo[™] Triplex Assay combines three existing Promega assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. The assay workflow occurs in two parts. The first part of the assay measures two protease biomarker activities (Figure 1); one is a marker of cell viability (live-cell), and the second is a marker of cytotoxicity (dead-cell). The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant substrate called GF-AFC. GF-AFC enters intact cells and is cleaved by the live-cell protease. Upon cleavage, a fluorescent signal is generated, which is proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic substrate called bis-AAF-R110 is used to measure dead-cell protease activity. Bis-AAF-R110, however, is cell-impermeant. Dead-cell proteases are released from cells that have lost membrane integrity. Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by viable cells.



Figure 1. Principle of the first step of the ApoTox-Glo[™] Assay. The biochemistry behind the first step of the ApoTox-Glo[™] Assay. Viability and cytotoxicity are measured in this step.

The live- and dead-cell proteases each produce a different fluorescent product, AFC and R110, respectively. These products have different excitation and emission spectra (Ex 400nm/Em 505nm and Ex 485nm/Em 520nm, respectively), allowing them to be detected simultaneously using a fluorescent plate reader.

The second part of the ApoTox-Glo[™] Triplex Assay detects caspase-3/7 activity using a luminogenic, caspase-3/7 substrate that contains the tetrapeptide sequence DEVD in a reagent optimized for caspase activity, luciferase activity and cell lysis (Figure 2). Adding the caspase-3/7 substrate reagent, which contains the Ultra-Glo[™] rLuciferase, results in cell lysis, intracellular caspase cleavage of the substrate and release of luciferin. The luciferin generates a luminescent signal produced by luciferase (1). The luminescent signal is proportional to the amount of caspase activity present and can be measured using a luminescent plate reader.



Figure 2. The biochemistry behind the second step of the ApoTox-Glo™ Assay. Caspase activity is measured in this step.

How to Perform the Assay

The ApoTox-Glo[™] Triplex Assay protocol uses a simple sequential "add-mix-read" format (Figure 3). The volumes of each assay component can be scaled to meet the varying throughput needs of researchers, and this assay is amenable to automation. Table 1 provides a description of the recommended volumes used at each assay step.



Figure 3. Overview of the ApoTox-Glo™ Triplex Assay protocol.

Recommended Cell Density (cells/well) Add cells + compound in culture medium	10,000-20,000	5,000-10,000	1 000-5 000	
Add cells + compound in culture medium			1,000-3,000	
	100µl	20µl	4μΙ	
	Treat cells at 37°C in 5% CO ₂ for the desired amount of time (i.e., 4–48 hours).			
Add Viability/Cytotoxicity Reagent	20µl*	5µl**	1.25µl***	
	Incubate at 37°C for 30 minutes.			
	Measure fluorescence at 2 wavelengths (400/50 and 485/520nm).			
Add Caspase-Glo® 3/7 Reagent	100µl	25µl	4µl	
	Incubate at room temperature for 30 minutes.			
	Measure luminescence.			

Table 1. ApoTox-Glo™ Triplex Assay protocol.

How the Assay Performs

Cell viability, cytotoxicity, and caspase-3/7 activity were assessed using the ApoTox-Glo[™] Triplex Assay with several different cell lines (K562, Jurkat, L929, and HepG2) treated for different periods of time with mechanistically different cytotoxic compounds (ionomycin, staurosporine, bortezomib, and SAHA) in 96-, 384-, and 1536-well assay plate formats. The cytotoxic profiles generated using the ApoTox-Glo[™] Triplex Assay measurements demonstrate the expected trends for each compound's effect on the cells. In vitro cytotoxicity is dependent upon compound dosage and exposure period. The kinetics of measurable cytotoxic biomarkers can vary widely between individual compounds and treatments. Therefore, it is important to characterize new compounds using multiple exposure periods(2).

lonomycin was selected because it causes primary necrosis in less than five hours, typically without caspase activation, and is a commonly used cytotoxic agent. Ionomycin is a bacterial ionophore used in research to affect calcium transport across cell membranes. Specifically, ionomycin stimulates production of inflammatory cytokines. Ionomycin treatment of in vitro cells for approximately 4–6 hours typically results in a dose-dependent decrease in cell viability and an increase in cytotoxicity without caspase-3/7 activation. This type of cellular profile is consistent with primary necrosis (Figure 4).



Figure 4. ApoTox-Glo[™] Triplex Assay with suspension K562 cells treated with ionomycin. Ionomycin treatment for 4 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with no caspase-3/7 activation, which is consistent with primary necrosis.

Similar to ionomycin, staurosporine also is considered a standard cytotoxic agent. Specifically, staurosporine is an antibiotic and pleiotropic ATP-competitive kinase inhibitor that causes caspase activation in cells after approximately 6–8 hours. Staurosporine acts to inhibit protein kinases by preventing the binding of ATP. Staurosporine treatment of in vitro cells for approximately 4–6 hours typically results in a dose-dependent decrease in cell viability, an increase in cytotoxicity, and an increase in caspase-3/7 activity consistent with apoptosis (Figure 5).



Figure 5. Apoptosis results using the ApoTox-Glo[™] Triplex Assay protocol. ApoTox-Glo[™] Triplex Assay with suspension Jurkat cells treated with staurosporine. Staurosporine treatment for 6 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis.

Bortezomib or Velcade[™] (Millennium Pharmaceuticals) is a proteasome inhibitor that is a new therapeutic treatment for multiple myeloma cancer. Bortezomib has been shown to inhibit proteasomes, cellular enzymes that play a role in regulation and cell growth processes, which in turn can lead to cancer cell death. Bortezomib treatment of in vitro cells for approximately 24 hours results similarly to staurosporine in a dose-dependent decrease in viability, an increase in cytotoxicity, and an increase in caspase-3/7 activity consistent with apoptosis (Figure 6).



Figure 6. ApoTox-Glo[™] Triplex Assay results for adherent HepG2 cells treated with bortezomib. Bortezomib treatment for 24 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis.

Finally, suberoylanilide hydroxamic acid (SAHA) or orinostat, rINN, or Zolinza[™] is a new anticancer therapeutic that has been shown to inhibit histone deacetylases (HDAC). SAHA treatment of in vitro cells for approximately 24–48 hours results in a dose-dependent decrease in cell viability, increase in cytotoxicity, and an increase in caspase activation events, consistent with apoptosis (Figure 7).



Figure 7. ApoTox-Glo[™] Triplex Assay results for suspension Jurkat cells treated with SAHA. SAHA treatment of Jurkat cells for 24 hours results in a dose-dependent decrease in viability, increase in cytotoxicity, and an increase in caspase-3/7 activity consistent with apoptosis.

Summary

Multiplexing with the Promega ApoTox-Glo[™] Triplex Assay helps to provide researchers with information about cell health, toxicity and apoptosis events, while minimizing the assay workflow time. The unique combination of chemistry successfully provides data assessing cell viability, cytotoxicity, and caspase activation events occurring inside the same population of cells. The assay is well suited for multi-well plate formats (96, 384, and 1536), and serves as an especially useful tool for academic researchers, biotechnology companies and secondary screening facilities working to better understand and predict the mechanism of cellular cytotoxicity.

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FIGURES



Figure 1. Principle of the first step of the ApoTox-Glo[™] Assay. The biochemistry behind the first step of the ApoTox-Glo[™] Assay. Viability and cytotoxicity are measured in this step.



Figure 2. The biochemistry behind the second step of the ApoTox-Glo™ Assay. Caspase activity is measured in this step.



Figure 3. Overview of the ApoTox-Glo™ Triplex Assay protocol.



Figure 4. ApoTox-Glo[™] Triplex Assay with suspension K562 cells treated with ionomycin. Ionomycin treatment for 4 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with no caspase-3/7 activation, which is consistent with primary necrosis.



Figure 5. Apoptosis results using the ApoTox-Glo[™] Triplex Assay protocol. ApoTox-Glo[™] Triplex Assay with suspension Jurkat cells treated with staurosporine. Staurosporine treatment for 6 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis.



Figure 6. ApoTox-Glo[™] Triplex Assay results for adherent HepG2 cells treated with bortezomib. Bortezomib treatment for 24 hours results in a dose-dependent decrease in viability and increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis.



Figure 7. ApoTox-Glo[™] Triplex Assay results for suspension Jurkat cells treated with SAHA. SAHA treatment of Jurkat cells for 24 hours results in a dose-dependent decrease in viability, increase in cytotoxicity, and an increase in caspase-3/7 activity consistent with apoptosis.

Tables

Table 1. ApoTox-Glo™ Triplex Assay protocol.				
	96-well	384-well	1536-well	
Recommended Cell Density (cells/well)	10,000-20,000	5,000-10,000	1,000-5,000	
Add cells + compound in culture medium	100µl	20µl	4µl	
	Treat cells at 37°C in 5% CO ₂ for the desired amount of time (i.e., 4–48 hours).			
Add Viability/Cytotoxicity Reagent	20µl*	5µl**	1.25µl***	
	Incubate at 37°C for 30 minutes. Measure fluorescence at 2 wavelengths (400/505nm and 485/520nm).			
Add Caspase-Glo® 3/7 Reagent	100µl	25µl	4µl	
	Incubate at room temperature for 30 minutes. Measure luminescence.			
* Combine 10μl of each substrate into 2ml ** Combine 10μl of each substrate into 2.5 *** Combine 10μl of each substrate into 3.	of the Viability/Cyto ml of the Viability/C 125ml of the Viabilit	toxicity Buffer. ytotoxicity Buffer. ty/Cytotoxicity Buffe	er.	

Table 1. ApoTox-Glo™ Triplex Assay protocol.



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