

IN VITRO TOXICOLOGY AND CELLULAR FATE DETERMINATION USING PROMEGA'S CELL-BASED ASSAYS

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This article describes options for measuring cellular fate by using reagents and systems developed to detect specific cellular markers and events. Here we show that a combination of Promega's cell-based assays provides cytotoxicity researchers the tools needed to differentiate cell fates such as apoptosis and necrosis.

Introduction

Cell viability determination is crucial for understanding cancer, apoptosis, cellular pathology, developmental mechanisms and pharmaceutical toxicity. To establish drug efficacy and safety, determining the fate of cells and the mechanisms behind their fates as they are exposed to pharmacological agents is critical. As a result, researchers need faster, more accurate, more economical and higher throughput methods for determining cytotoxicity.

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Current models suggest that mitochondria act as exquisite biosensors of cell viability. In particular, mitochondrial membranes respond to relatively low concentrations of toxic compounds, leading to a cascade of biochemical signals that culminates in the release of cytochrome c and the progression of apoptosis. This form of cell death limits damage to adjacent cells, since there is little or no leakage of cellular contents. In vitro, a toxic compound may initiate apoptosis, eventually progressing to loss of membrane integrity and leakage of cellular components, which are characteristic of secondary necrosis. At extremely high doses, these compounds may destroy the cell membrane so quickly that apoptotic pathways cannot be initiated.

The precise transition between apoptosis and necrosis remains indistinct. In some cases, toxicity presents initially as apoptosis, but as the membrane damage increases, the cells can become necrotic. In this situation, both apoptotic and necrotic markers may be present, depending on the timing of the assay relative to when the cell was exposed to the toxic compound.

Tools Used

CellTiter-Glo™ Luminescent Cell Viability Assay^(a) is designed to monitor cytotoxicity as well as cell proliferation by measuring the number of viable cells in culture. The assay measures the amount of ATP present in culture, which is

directly proportional to the number of viable cells. The assay generates a glow-type luminescent signal, and the “add, mix and read” format allows for multiple throughput formats.

CytoTox 96® Non-Radioactive Cytotoxicity Assay is a colorimetric alternative to radioactive cytotoxicity assays. The assay simply measures the release of lactate dehydrogenase (LDH) into culture medium upon cell lysis. LDH is measured by a coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The absorbance at 490nm is directly proportional to the number of lysed cells.

Apo-ONE™ Homogeneous Caspase-3/7 Assay^(b) provides for fast and sensitive detection of caspase-3 and -7 using an “add, mix and read” format. These caspases play key effector roles in apoptosis through cleavage of specific substrates important for downstream apoptotic events. The Homogeneous Caspase-3/7 Buffer rapidly lyses mammalian cells and supports optimal caspase-3/7 activity. The supplied substrate (Z-DEVD-rhodamine 110) is cleaved at the DEVD peptide when caspase-3/7 is present, resulting in release of rhodamine 110 (R110). The R110 leaving group is intensely fluorescent when excited at 499nm (emission maximum is 521nm).

Beckman Coulter's Biomek® 2000 Laboratory Automation Workstation is a medium-throughput automation platform capable of performing these assays through liquid handling, plate movement and integrated shaking. For staurosporine-treated cultures, all three assay types were set up on the Biomek® 2000 workstation with downstream plate reading using offline readers specific for the three assay outputs (luminescent, fluorescent and colorimetric).

A combination of Promega's cell-based assays provides cytotoxicity researchers the tools needed to differentiate cell fates such as apoptosis and necrosis.

Methods

The human hepatocyte HepG2 cell line was used for all assays. The cells were initially plated in 96-well black tissue culture plates with a clear bottom (Corning #3603) at 40,000 cells/well. The cells were allowed to grow to confluence before treatment. To demonstrate apoptosis and cytotoxicity, cells were treated with a range of 48nM to 25µM staurosporine for 24 or 48 hours (or the corresponding DMSO vehicle control). To demonstrate necrosis, cells were treated with 1%, 5%, or 10% Triton® X-100 and assayed manually. All plates and assay reagents

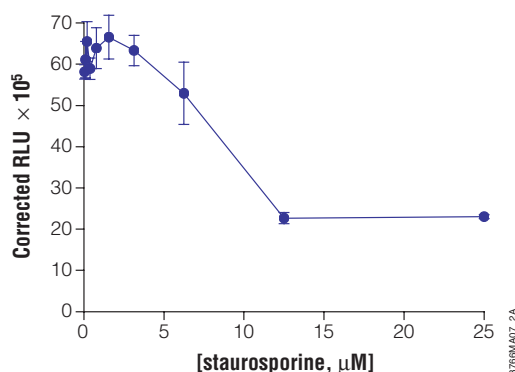


Figure 1. The CellTiter-Glo™ Assay detects a dose-dependent decrease in cell viability in staurosporine-treated cultures. Dosage range of 48nM to 25 μM shows a decrease in cell viability with increasing drug dosage. Plotted data are corrected for vehicle-only control values.

were allowed to equilibrate to room temperature before starting the assays.

For CellTiter-Glo™ Assays of staurosporine-treated cultures, the Biomek® 2000 workstation dispensed the CellTiter-Glo™ Reagent and then agitated the plates for two minutes on the integrated shaker. Plates were incubated for 10 minutes in the dark at room temperature before being read on an EG&G MicroLumat Luminometer.

Apo-ONE™ Assays of staurosporine-treated cultures were set up in the same manner as the CellTiter-Glo™ Assays. After shaking, plates were incubated for two hours in the dark at room temperature before being read on a BMG POLARStar fluorescent plate reader.

CytoTox 96® Non-Radioactive Cytotoxicity Assays of staurosporine-treated cultures were set up on the Biomek® 2000 workstation. For these experiments, the robot dispensed 25 μl of water (necessary to dilute the strong reaction) and 50 μl of CytoTox 96® Reconstituted Substrate Mix to a new 96-well reaction plate (Corning #3596). The robot then removed 25 μl of medium from the culture plate, added the medium to the reaction plate and mixed by pipetting. Plates were manually covered with foil and allowed to incubate for 15 minutes. The robot then added 50 μl of Stop Solution. Absorbance was recorded using a Molecular Devices SpectraMAX® spectrophotometer.

To demonstrate necrosis (defined as immediate lysis of cells and LDH release with no caspase-3/7 detection), one column of cells in each of two separate plates was treated with 1%, 5% or 10% Triton® X-100 in medium or medium

alone for approximately two minutes. Both the CytoTox 96® Non-Radioactive Cytotoxicity Assay and Apo-ONE™ Homogeneous Caspase-3/7 Assay were set up manually in a similar fashion as the Biomek® 2000 protocols. CytoTox 96® Assays were read after a 15-minute incubation with CytoTox® 96 Reconstituted Substrate Mix. Apo-ONE™ Assays were read 10 minutes, 1 hour and 2 hours after Apo-ONE™ Reagent addition.

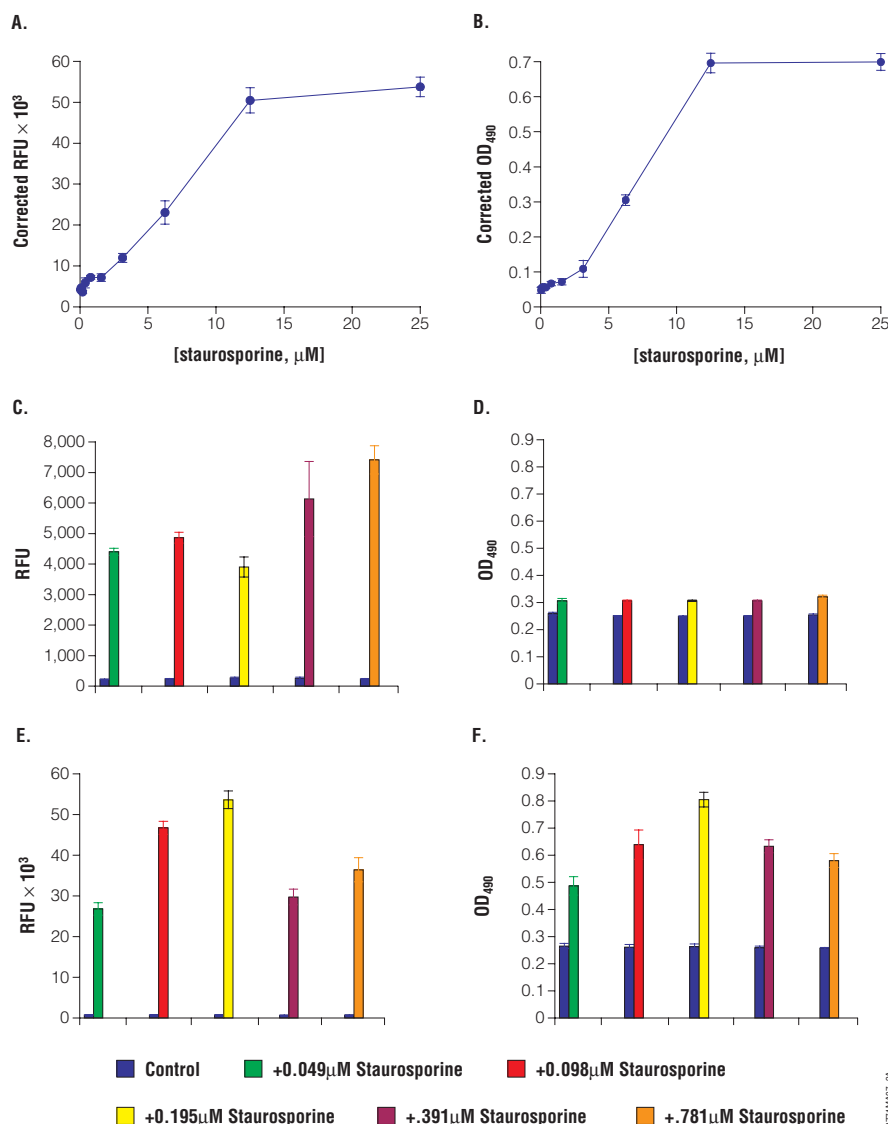
Results

CellTiter-Glo™ Assay results show that, at high concentrations of staurosporine (12.5 μM and 25 μM), cell viability is severely affected over the 24-hour drug treatment (Figure 1). The effect becomes less severe at lower doses.

Apo-ONE™ Assay and CytoTox 96® Assay data demonstrate apoptosis as well as loss of membrane integrity at staurosporine levels at or above 3.125 μM . The cytotoxic effect is more obvious at concentrations at or above 6.25 μM (Figure 2A and 2B). However, at doses at or below 781nM, caspase-3/7 activity remains very high relative to the corresponding vehicle-only controls (Figure 2C and 2D), but the cytotoxic effect is minimal. These data demonstrate that the lower concentrations of staurosporine initiate the caspase-3/7 apoptotic pathway but do not result in detectable loss of membrane integrity (secondary necrosis) under these assay conditions (i.e., measured at this time interval). However, after 48 hours (Figures 2E and 2F), as caspase-3/7 levels remain high relative to control, CytoTox 96® Assay signals are also high relative to 24-hour treatment (Figure 2D) and to the corresponding vehicle-only controls.

Apo-ONE™ and CytoTox 96® Assay results demonstrate apoptosis as well as loss of membrane integrity.

Cultures treated with the dosage range of Triton® X-100 and assayed with both Apo-ONE™ Assay and CytoTox 96® Assay demonstrate time- and dosage-dependent responses. Figure 3 shows the ratio of Triton® X-100 signal to signal obtained from medium alone for both assays. Note that the ratios for Apo-ONE™ Assays start at 1.0 for 10% Triton® X-100, meaning that the signal from Triton® X-100 equalled that from medium alone. The CytoTox 96® Assay ratio at this same time point is 8.4. This suggests that high concentrations of Triton® X-100 cause cell lysis (necrosis) without apoptosis. However, at 1.0% Triton® X-100, the ratio for Apo-ONE™ climbs to 40, demonstrating strong caspase-3/7 response while the CytoTox 96® Assay ratio is 4.6. Perhaps the high concentration of detergent affects enzyme function directly. To explore this possibility,



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Figure 2. Apo-ONE™ Assay and CytoTox 96® Assay detect apoptosis and cytotoxicity in staurosporine-treated cultures.

Panel A. Treatment with a dosage range of up to 25 μM staurosporine for 24 hours indicates induction of apoptosis as determined by the Apo-ONE™ Assay. **Panel B.** Cytotoxicity for the same dosage range (24-hour treatment) of staurosporine as assayed using the CytoTox 96® Assay. The EC₅₀ values calculated using both assays are comparable. CytoTox 96® Assay EC₅₀ = 7.005; Apo-ONE™ Assay EC₅₀ = 7.382. **Panel C.** Low doses of staurosporine (48–781 nM; 24-hour treatment) also show a significant effect on caspase-3/7 activity compared to the corresponding vehicle-only controls. **Panel D.** Low doses of staurosporine (48–781 nM) show only minimal or no LDH release after 24 hours. **Panel E.** After 48-hour treatments with 48–781 nM staurosporine, the Apo-ONE™ Assay continues to detect significant caspase activity. **Panel F.** LDH release measured using the CytoTox 96® Assay shows an increase in LDH release after 48-hour treatments compared to the minimal release observed in shorter treatments (Panel D) and to the corresponding vehicle-only control. Staurosporine was dissolved in DMSO. The vehicle-only controls reflect the increase in DMSO concentration required to dissolve the increasing concentrations of staurosporine. Data in Panels A and B are corrected for vehicle-only control values. The legend for Panels C–F is provided at the bottom of the figure.

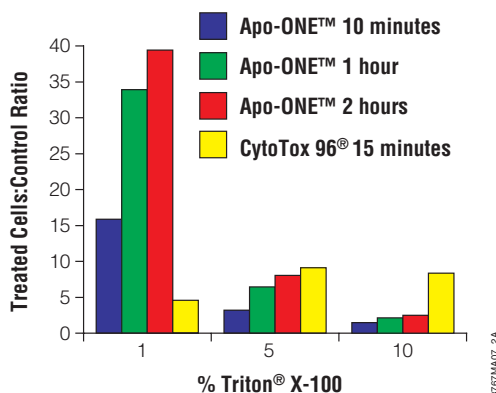


Figure 3. The ratio of signal from Triton® X-100-treated cells to signal obtained from cells in medium alone for both Apo-ONE™ Assay and CytoTox 96® Assay.

concurrent experiments examined the effect of Triton® X-100 on purified caspase-3 alone or caspase-3 spiked into Triton® X-100-treated cells. The data (not shown) indicated a reduction in caspase activity (approximately 50%) at 10% Triton® X-100 treatment after a two-hour incubation with Apo-ONE™ Reagent. These experiments hint that the lack of Apo-ONE™ Assay signal at high Triton® X-100 concentrations may be due to an artificial mechanism interfering with the assay. However, regardless of mechanism, this experiment shows that timing and dosage play a critical role in developing and optimizing drug toxicity experiments as well as in interpretation of cellular fates.

Conclusion

We show that combining a homogeneous luminescent ATP assay, a homogeneous fluorescent caspase-3/7 assay and a colorimetric LDH assay integrated on a robotic workstation gives researchers the tools needed to differentiate among the various cell fates associated with toxicity. We demonstrated that timing and dosage are critical to assay development and results interpretation.

Protocols

Apo-ONE™ Homogeneous Caspase-3/7 Assay Technical Bulletin, #TB295

www.promega.com/tbs/tb295/tb295.html

CellTiter-Glo™ Luminescent Cell Viability Assay Technical Bulletin, #TB288

www.promega.com/tbs/tb288/tb288.html

CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin, #TB163

www.promega.com/tbs/tb163/tb163.html

Ordering Information

Product	Size	Cat.#
Apo-ONE™ Homogeneous Caspase-3/7 Assay ^(b)	1ml	G7792
	10ml	G7790
	100ml	G7791
CellTiter-Glo™ Luminescent Cell Viability Assay ^(a)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CytoTox 96® Non-Radioactive Cytotoxicity Assay*	1,000 assays	G1780

*For Laboratory Use.

(a)Patent Pending.

(b)This product is covered by U.S. Pat. Nos. 4,557,862 and 4,640,893 and is sold for research use only. All other uses, including but not limited to use as a clinical diagnostic or therapeutic, require a separate license. Please contact Promega Corporation for details relating to obtaining a license for such other use.

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