

IN VITRO CYTOTOXICITY SCREENING OF HUMAN NEUROBLASTOMA CELLS

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Introduction

Robotic integration of cell-based assays for in vitro toxicity screening of chemical compounds is becoming an increasingly important aspect of the drug discovery process. Development of assays that simplify the automation process is crucial for the success of this high-throughput screening. Promega has recently introduced the CytoTox-ONE™ Homogeneous Membrane Integrity Assay^(a), a fluorometric method for estimating the number of non-viable cells present in multiwell plates. Combining this assay with Promega's Apo-ONE™ Homogeneous Caspase-3/7 Assay^(b) provides researchers with valuable insight into the mechanisms of cellular toxicity.

The human SH-SY5Y cell line is a pure neuroblastoma subclone of the cell line, SK-N-SH, derived by Dr. June Biedler (Sloan-Kettering Institute for Cancer Research, Rye, NY, USA) (1,2). These cells are used as experimental models in neurobiology to study cell differentiation and cell signaling, neurotransmission and ion channel function, molecular mechanisms of drug addiction and neurodegenerative diseases, neurotoxicity, and neuropharmacology (3–8).

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay is a fluorometric method for estimating the number of non-viable cells.

This article shows the compatibility of the SH-SY5Y cell line with higher throughput toxicology experiments using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay and Apo-ONE™ Homogeneous Caspase-3/7 Assay on a robotic platform. Briefly, we performed a Z' factor analysis on SH-SY5Y cells in a 96-well format, exposing half of each plate to 3.125µM staurosporine and the other half of the same plate with the appropriate DMSO vehicle control.

Tools Used

CytoTox-ONE™ Homogeneous Membrane Integrity Assay is a rapid, homogeneous fluorescent measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH release into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin as shown in Figure 1. Since the CytoTox-ONE™ Reagent mix does not damage healthy cells, the reaction can be performed directly in the wells containing the mixed population of damaged and healthy cells.

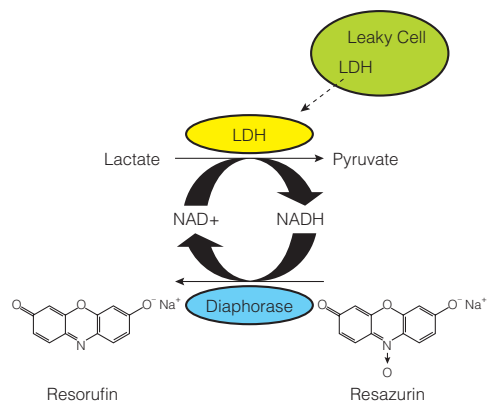


Figure 1. CytoTox-ONE™ Homogeneous Membrane Integrity Assay. Release of LDH from damaged cells is measured by supplying lactate, NAD⁺, and resazurin as substrates in the presence of diaphorase. Generation of the fluorescent resorufin product is proportional to the amount of LDH.

Apo-ONE™ Homogeneous Caspase-3/7 Assay provides for fast and sensitive detection of caspase-3 and -7 in an "add, mix and read" format. These caspases play key effector roles in apoptosis through cleavage of specific substrates important for downstream apoptosis signaling. 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 with an emission maximum of 521nm.

Beckman Coulter's Biomek® 2000 Laboratory Automation Workstation is a medium-throughput automation platform capable of setting up these assays through liquid handling, plate movement and integrated shaking. For staurosporine-treated cultures, both assay types were set up on the Biomek® 2000 workstation with downstream plate reading performed on an offline plate reader.

Methods

SH-SY5Y neuroblastoma cells obtained from ATCC (CRL-2266) were cultured as described previously (4). Briefly, cells were cultured in a 1:1 mixture of Ham's F12 nutrients and minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100IU/ml penicillin, and 100mg/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C.

Cells were allowed to reach approximately 70% confluence. They were then trypsinized from the flask and plated in 96-well black, clear-bottom, tissue culture-treated plates (Corning #3603) in 45% MEM, 45% F12K, 10% FBS. After

48 hours, one half of the plate was treated with 100µl 3.125mM staurosporine in DMSO and medium and the other half with the appropriate DMSO and medium vehicle control. The cells were treated for 24 hours prior to assays.

Culture plates and CytoTox-ONE™ Assay Buffer were allowed to equilibrate to room temperature. To make the CytoTox-ONE™ Reagent, 11ml of Assay Buffer was added to the lyophilized Substrate Mix. To perform the CytoTox-ONE™ Assay, a two-plate protocol was written for Beckman Coulter's Biomek® 2000 workstation. The robot added 100µl of CytoTox-ONE™ Reagent to each well followed by a short (30-second) mix on the integrated shaker. Plates were allowed to incubate for 10 minutes after which the robot added 50µl of CytoTox-ONE™ Stop Solution. To distribute the Stop Solution evenly, the plates were briefly agitated on the integrated shaker. For all experiments, plates were immediately read on a BMG POLARStar fluorescent plate reader equipped with a 544nm excitation and 620nm emission filter set.

For Apo-ONE™ Homogeneous Caspase-3/7 assays on staurosporine-treated cultures, the Biomek® 2000 dispensed 100µl of Apo-ONE™ Buffer followed by plate shaking for two minutes on the integrated shaker. The plates were allowed to incubate for two hours in the dark at room temperature before being read on a BMG POLARStar fluorescent plate reader.

Results

To determine assay robustness and reproducibility of the system, the statistical calculation for Z' factor was used (9). This "screening window coefficient" compares the assay dynamic range to data variation making it a powerful tool to assess assay quality. Z' factor for each plate processed by the Biomek® 2000 workstation was calculated using the following formula:

$$1 - \frac{(3*SD \text{ of sample} + 3*SD \text{ of control})}{|(\text{mean of sample} - \text{mean of control})|}$$

A Z factor equal to 1.0 is a perfect assay. Z factors greater than or equal to 0.5 indicate an excellent assay. Figure 2A represents an assay with Z' factor of 0.79 for one of the CytoTox-ONE™ Assays processed by the Biomek® 2000 workstation. Figure 2B shows a Z' factor of 0.74 for an Apo-ONE™ Assay. This indicates that the system used (the combination of Biomek® 2000 processing and Promega's cell-based assays) provides for excellent assays.

References

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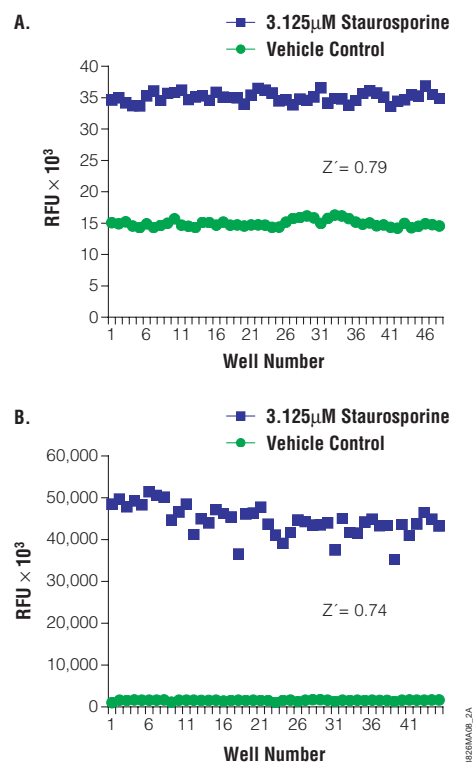


Figure 2. Z' factor analysis of staurosporine-treated SH-SY5Y cells compared to vehicle control. Panel A. CytoTox-ONE™ Assay results from one plate show a Z' factor of 0.79, n = 48. **Panel B.** Apo-ONE™ Assay results show a Z' factor of 0.74, n = 45.

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Ordering Information

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay ^(a)	200–800 assays	G7890
	1,000–4,000 assays	G7891
Apo-ONE™ Homogeneous Caspase-3/7 Assay ^(b)	1ml	G7792
	10ml	G7790
	100ml	G7791

^(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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