Introducing the CytoTox-ONE™ Homogeneous Membrane Integrity Assay

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To assist researchers in measuring cell viability, Promega introduces the CytoTox-ONE™ Homogeneous Membrane Integrity Assay. This article describes the assay and its advantages in cytotoxicity screening.

In vitro cytotoxicity assays detect the degree to which a treatment is toxic to cells by measuring the viability of a population of cells in culture. Cell viability can be reflected by a variety of different parameters, but it is most often defined experimentally by the integrity of the outer cell membrane. If the cell membrane is full of "holes", it allows substances to move across the cell membrane that would normally be excluded. Vital dyes such as trypan blue or propidium iodide are usually excluded from viable cells, but they can enter through holes in damaged cell membranes and stain the cytoplasm and therefore can be used as an indicator of nonviable cells. Measuring the leakage of components out of the cytoplasm through holes in damaged cell membranes can also indicate loss of membrane integrity in both in vivo and in vitro experimental situations.

The recently introduced CytoTox-ONE™ Homogeneous Membrane Integrity Assay[a] is a fluorometric method for estimating the number of nonviable cells. The CytoTox-ONE™ Assay measures the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into the fluorescent compound, resorufin, as shown in Figure 1.

The homogeneous format of the CytoTox-ONE™ Assay is ideally suited for automated in vitro cytotoxicity screening.

The CytoTox-ONE™ Reagent was designed so that it does not damage healthy cells in vitro; therefore, the assays to measure released LDH can be performed directly in tissue culture wells containing a mixed population of viable and damaged cells. The homogeneous format of the CytoTox-ONE™ Assay is ideally suited for automated in vitro cytotoxicity screening in 96- and 384-well plates (1).

The product is supplied as a lyophilized Substrate Mix that the user reconstitutes with Assay Buffer to form the CytoTox-ONE™ Reagent. A flow diagram summarizing the assay protocol is shown in Figure 2. Assay plates are allowed to equilibrate to ambient temperature; then CytoTox-ONE™ Reagent is added to each well and incubated for 10 minutes. Stop Solution is added, and the fluorescent signal is measured. Fluorescence data are collected using a standard 96- or 384-well fluorometer. A 560nm excitation and 590nm emission filter set is recommended. The amount of fluorescence produced is proportional to the number of lysed cells using a 96- or 384-well format (Figure 3).

Methods for determining LDH release in conjunction with diaphorase have been used for several years. Variations on this technology have been reported for measuring natural

Assay Benefits

Use a widely accepted parameter to measure viability
The assay results indicate cell membrane integrity, the parameter most often used to define cell viability.

Assay directly in cell culture plates
The assay uses a homogeneous method; reagent is added directly to cultured cells.

Obtain results quickly
The procedure is faster than colorimetric LDH assay methods that require transfer of an aliquot of culture supernatant to a separate enzymatic assay plate.

Choose assay format
The protocol is compatible with 96- and 384-well formats.

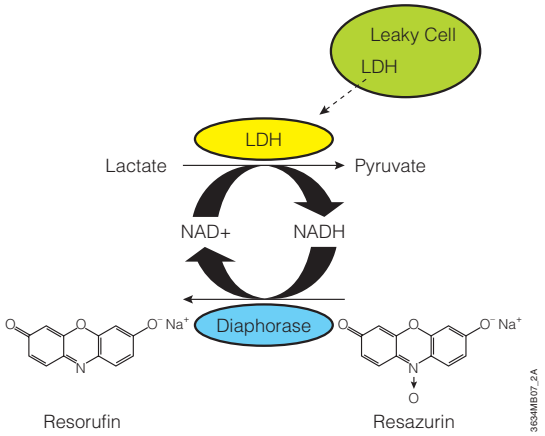

Figure 1. Measuring cell viability using the CytoTox-ONE™ Assay. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay measures the release of LDH from cells using a coupled enzymatic assay that results in the conversion of resazurin into the fluorescent compound, resorufin.

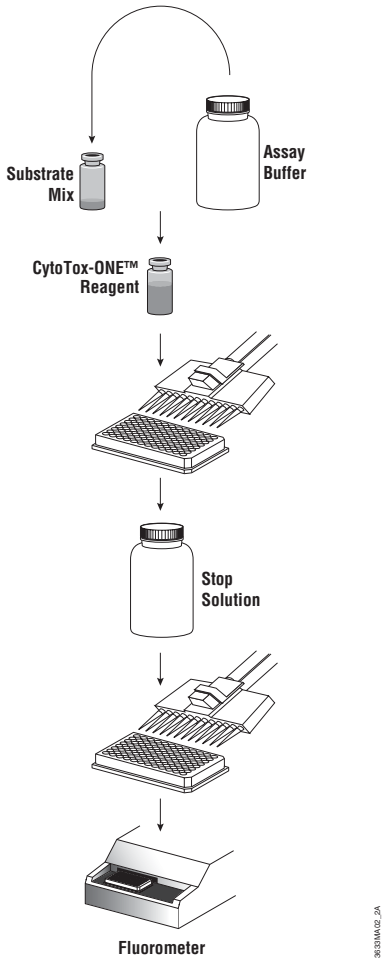

Figure 2. The CytoTox-ONE™ Assay protocol. Assay plates are allowed to equilibrate to ambient temperature, and CytoTox-ONE™ Reagent is added to each well and incubated for 10 minutes. Stop Solution is added, and the fluorescent signal is measured.

cytotoxicity and have been demonstrated to be identical within experimental error to values determined in parallel ^{51}Cr release assays (2,3).

The rationale for choosing LDH rather than another cytosolic enzyme is that LDH activity is universally present in eukaryotic cells and that the enzymatic activity is more stable than other candidates such as glucose-6-phosphate dehydrogenase (Figure 4). Measuring an enzyme with a long half-life is an advantage when considering the design of cytotoxicity assay protocols, especially if cells are exposed to the test compound for a long period of time.

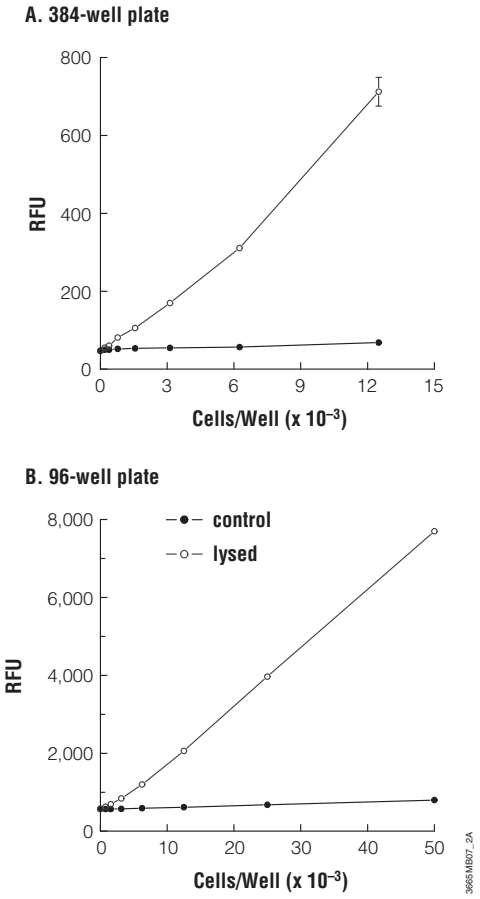

Figure 3. Linear relationship ($r^2 > 0.95$) between cell number and fluorescence ($560_{Ex}/590_{Em}$) using the CytoTox-ONE™ Assay in 384-well and 96-well plate formats. Panel A. Serial two-fold dilutions of L929 cells were made in a 384-well plate containing 25µl of serum-supplemented medium per well. **Panel B.** Serial two-fold dilutions were made in a 96-well plate containing 100µl medium per well. Wells were treated by addition of Triton® X-100 to produce "lysed" cells. Wells containing control cells received PBS as the vehicle control. CytoTox-ONE™ Reagent was added following treatments. Values represent the mean ± S.D. from 4 replicate wells for each cell number. The lowest values shown for each plate format (195 cells for 384-well format; 781 cells for 96-well format) are significantly different than the "zero" cell background fluorescence.

For example, if the released component has a two-hour half life, it would be difficult to distinguish between complete cell lysis occurring at time zero versus 25% cell lysis at 4 hours.

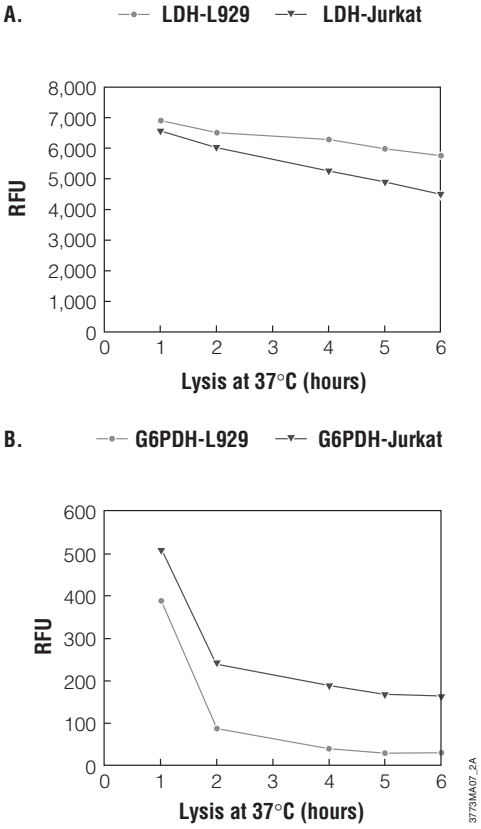

Figure 4. Stability comparison of two cytoplasmic enzymes. L929 and Jurkat cells were cultured in a 96-well plate prior to staggered additions of detergent to lyse cells. Between each addition, cells were maintained at 37°C, 5% CO_2. **Panel A.** To monitor relative stability of lactate dehydrogenase and to conclude the experiment, CytoTox-ONE™ Reagent was added to one series of lysed cells. **Panel B.** To measure glucose-6-phosphate dehydrogenase in parallel, a modified reagent was prepared to contain glucose-6-phosphate and NADP+ as well as diaphorase and resazurin. With subtraction of the "no-cell" background fluorescence, the stability of each enzyme for both cell types can be seen.

The LDH release assay is commonly used for testing cytotoxicity of various experimental compounds. Figure 5 shows data generated using the CytoTox-ONE™ Assay and depicts the cytotoxic effect of TNFα on murine L929 cells. Increasing concentrations of TNFα are toxic to L929 cells, resulting in a loss of membrane integrity, release of LDH into the surrounding medium, and a greater fluorescent signal. Figure 5 also shows the results obtained using the CellTiter-Glo™ Luminescent Cell Viability Assay[a] to measure ATP content, which estimates the number of

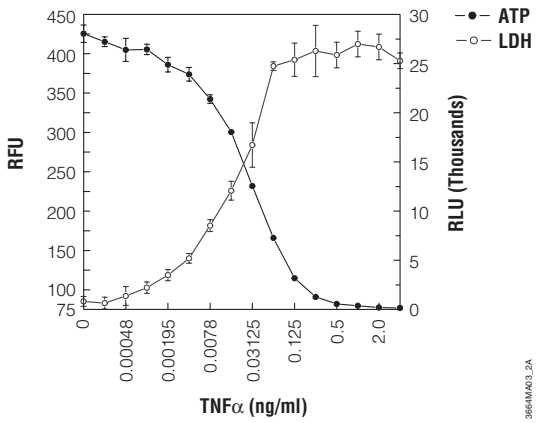

Figure 5. Murine L929 cells were seeded at 2,000 cells per well in a 384-well plate in serum-supplemented medium, cultured for 24 hours, then various amounts of TNFα were added and incubated overnight. Either CytoTox-ONE™ Reagent or CellTiter-Glo™ Reagent was added to parallel sets of wells, and fluorescence ($560_{Ex}/590_{Em}$) or luminescence values, respectively, were recorded. (The CellTiter-Glo™ Assay uses ATP content to indicate the number of viable cells.) The values represent the mean ± SD of 4 replicate samples. The half maximal response values correlate well for the cytotoxicity and viability assays.

viable cells (4,5). Concentrations of TNFα that are toxic to L929 cells result in a loss of ATP and a reduced luminescent signal. The IC_{50} values determined using both assays are similar.

The CytoTox-ONE™ Assay can be combined with other assay methods to collect data for more than one parameter from the same experimental sample. It is possible to remove a portion of the medium from each well to a separate fluorescence-compatible multiwell plate to measure LDH release using the CytoTox-ONE™ Assay in addition to performing a separate assay on the sample remaining in the original well. One advantage of this approach is that any type of multiwell plate can be used to culture cells, providing more flexibility for specific secondary assay formats. Some examples of this application might include measuring reporter gene expression or confirming the number of viable cells by measuring the ATP content using the CellTiter-Glo™ Assay or by measuring tetrazolium reduction using the CellTiter 96® AQ_{ueous} One Solution Assay[b]. Alternatively, the level of apoptosis could be determined by measuring the caspase activity using the Apo-ONE™ Homogeneous Caspase-3/7 Assay[c].

The CytoTox-ONE™ Assay also can be used to determine the total number of cells present in wells at the end of a

proliferation assay. The procedure involves lysing all the cells to release total LDH and then adding the CytoTox-ONE™ Reagent. The total number of cells present will be directly proportional to the background-subtracted fluorescence values, which represent LDH activity (6). An example demonstrating this application can be seen in the "lysed" data shown.

A detailed experimental protocol and additional discussion of general considerations for the CytoTox-ONE™ Assay can be found in Technical Bulletin #TB306. Those considerations include: background fluorescence/serum LDH, temperature, assay controls, considerations for measuring maximum LDH release, light sensitivity of resazurin, use of Stop Solution to stop development of fluorescent signal, and cell culture media considerations.

References

1. *CellTiter 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin* #TB163, Promega Corporation.
2. Korzeniewski, C. and Callewaert, D.M. (1983) An enzyme-release assay for natural cytotoxicity. *J. Immunol. Meth.* **64**, 313–20.
3. Decker, T. and Lohmann-Matthes, M.L. (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Meth.* **115**, 61–9.
4. *CellTiter-Glo™ Luminescent Cell Viability Assay Technical Bulletin* #TB288, Promega Corporation.
5. Crouch, S.P. *et al.* (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxity. *J. Immunol. Meth.* **160,** 81–88.
6. Moravec, R. (1994) Total cell quantitation using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. *Promega Notes* **45**, 11–12.

Protocol

CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin #TB306

www.promega.com/tbs/tb306/tb306.html

Ordering Information

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
	1,000–4,000 assays	G7891

(a)Patent Pending.
(b)The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.
(c)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.

Promega Scientists will Present Work at 3rd International Symposium on Early Toxicity Screening

Promega scientists will participate in the 3rd International Symposium on Early Toxicity Screening sponsored by The Institute for Scientific Exchange, Inc. The symposium, which focuses on Early ADME-Tox Screening Approaches, is designed to foster communication among industrial, regulatory and academic practictioners.

"Apoptosis vs. Cytotoxicity vs. Necrosis: Use of Cell-Based Assays to Distinguish Between Different Mechanisms of In Vitro Toxicity" will discuss the use of automated cell-based assays for in vitro toxicity screening of chemical compounds. Promega's automatable and homogeneous cell-based assays for measuring cellular metabolism using ATP, identifying apoptosis by measuring caspase-3/7 activity, and determining cell viability through measuring LDH release will be discussed.

In the afternoon, Promega scientist, Jim Cali, Ph.D., will present "Novel Probe Substrates for Drug-Metabolizing Cytochrome p450s." Cytochrome p450s play a central role in drug metabolism and safety, and understanding p450/drug interactions can be predictive of drug disposition in patients. Cali will describe novel cytochrome p450 substrates that can be used in scalable assays.

For more information about the conference, visit the Institute for Scientific Exchange web site at www.isciencex.com

3rd International Symposium on Early Toxicity Screening: Early ADME-Tox Screening Approaches

December 11, 2002
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