Abstract
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.

Introduction
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 to 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 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 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).

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.
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 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 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. 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 37% cell lysis at 4 hours.

**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.

**Figure 3. Linear relationship ($r^2 > 0.95$) between cell number and fluorescence (560Ex/590Em) 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 25 µl of serum-supplemented medium per well. Panel B. Serial two-fold dilutions were made in a 96-well plate containing 100 µl medium per well. Wells were treated by adding 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.
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 to measure ATP content, which estimates the number of viable cells. Concentrations of TNFα that are toxic to L929 cells result in a loss of ATP and a reduced luminescent signal. The IC₅₀ values determined using both assays are similar.

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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® AQueous 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 followed by 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 (d). An example demonstrating this application can be seen in the "lysed" data shown in Figure 3.
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

Protocol

Ordering Information

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*Patent Pending.*

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