Homogeneous Fluorescent *In Vitro* Toxicity Assay that Measures Cell Membrane Integrity

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Abstract

We have developed a homogeneous fluorescent cytotoxicity assay that measures the release of lactate dehydrogenase (LDH) from cells with damaged membranes. The novel "add and read" homogeneous format of the CytoTox-ONE™ Assay (patent pending) was enabled by developing assay conditions that do not damage living cells, thus only LDH present in culture medium is detected and viable cells do not contribute to the signal. The CytoTox-ONE™ Assay procedure is to add reagent directly to cells, incubate for 10 min, stop the reaction, and record fluorescence. There is a linear relationship between the fluorescent signal and the number of damaged cells. The assay can detect fewer than 200 damaged cells in a 384 well format. The release of LDH from cells correlates inversely with viable cell number. The stability of the reagents and fluorescent signal are compatible with automation of the assay for HTS. Multiplexing of the CytoTox-ONE™ Assay with other assays will be described.

Linearity and Sensitivity of CytoTox-ONE™ Assay in 384 well Plates

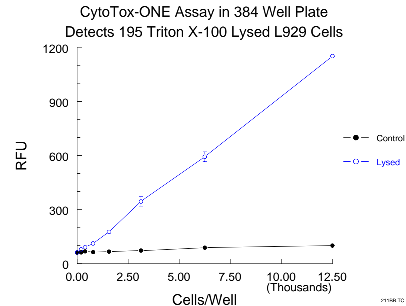


Fig. 1. Serial dilutions of L929 cells in F12/DME + 10% horse serum were made in a 384 well white walled clear bottom plate (22.5µl per well). Cells were cultured for 1.5hr at 37°C, then lysed by addition of 2.5µl per well 2% Triton X-100. Intact cells received PBS as a control. The plate was shaken and equilibrated to ambient temperature. CytoTox-ONE Reagent (25µl per well) was added and shaken for 30 sec. The plate was incubated for 10 min, then 12.5µl Stop Solution added. Fluorescence $560nm_{Ex}/590nm_{Em}$ was recorded on a Labsystems Fluoroscan Ascent plate reader.

Reconstituted CytoTox-ONE™ Reagent is Stable for Several Weeks

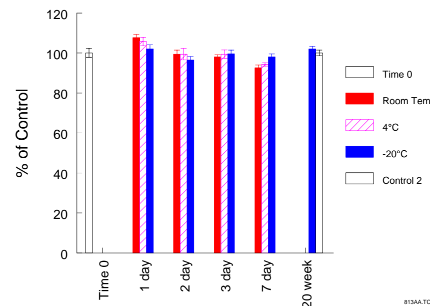


Fig. 4. The reconstituted CytoTox-ONE™ Reagent is stable for least 20 weeks (longest time tested) when stored at –20°C protected from light. The Reagent shows negligible loss of activity when stored at 4°C or room temperature for up to 7 days. A large quantity of Reagent was prepared and pooled at time 0 and placed at ambient, 4°C and –20°C. The quality control test was performed at each time point as indicated on the x-axis. Fresh of Reagent was prepared and run as Control 2 for the 20 week sample.

Use of Serum-Free Medium Reduces Background Fluorescence Caused by LDH Contamination in Serum

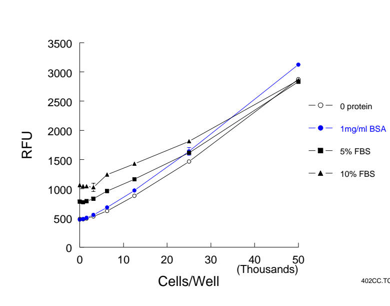


Fig. 7. Serum naturally contains LDH activity which contributes to background fluorescence in the CytoTox-ONE™ Assay. Notice the effect of 0%, 5%, & 10% FBS on background fluorescence in the "no cell" controls. Serial dilutions of Jurkat cells were prepared in RPMI 1640 containing 0%, 5%, and 10% FBS. Cells were treated with Lysis Solution, and the CytoTox-ONE™ Assay was incubated for 10 min. LDH in serum was not destroyed by traditional heat inactivation at 56°C.

Multiplex use of CytoTox-ONE™ and Apo-ONE™ Assays on the Same Samples

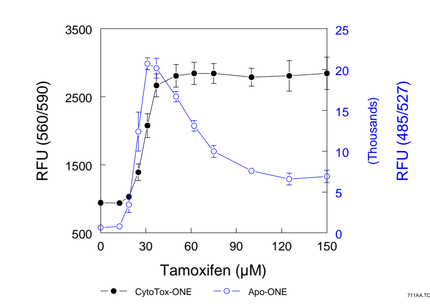
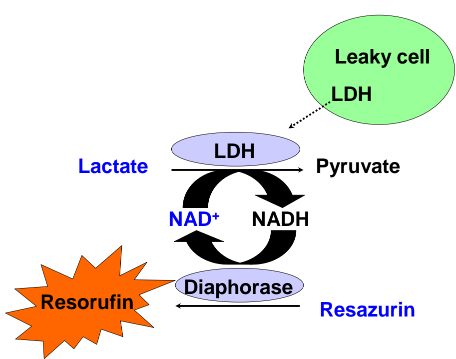


Fig. 10. HepG2 cells (10,000 cells/well) were cultured overnight in a solid white, clear bottom 96-well plate. Tamoxifen was added to wells and incubated for 4hr at 37°C. To assay LDH activity, 50µl/well culture supernatants were transferred to a 96-well plate containing 50µl/well of CytoTox-ONE Reagent and incubated at ambient temperature for 30 min prior to addition of 25µl/well Stop Solution and recording fluorescence (560/590nm). For caspase-3/7 determination, 50µl/well of Apo-ONE™ Reagent were added to the original culture plate containing cells, and incubated at ambient temperature for 45 min prior to recording fluorescence $485nm_{Ex}/527nm_{Em}$.

CytoTox-ONE™ Assay Mechanism Overview



Long Half-Life of LDH Provides an Advantage for Cytotoxicity Assays

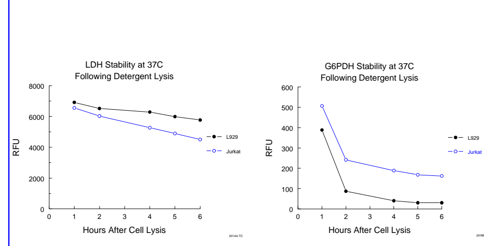


Fig. 2. LDH is more stable than G-6-PDH once release from cells. L929 cells (10,000 cells) in F12 /DME + 10% horse serum and Jurkat cells (30,000 cells) in RPMI 1640 + 10% FBS were prepared in a 96 well plate and cultured overnight. Staggered additions of detergent were made the following day over time, with the plate returned to 37°C after each addition. LDH activity using the CytoTox-ONE Reagent and glucose-6-phosphate dehydrogenase activity using a similar substrate modified reagent were determined.

The Fluorescent Signal is Stable for Days After Stop Solution is Added

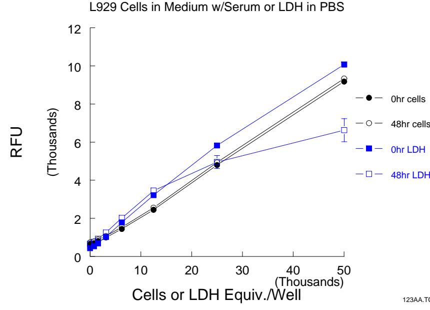


Fig. 5. The fluorescent signal from samples of cells was stable for 48 hours after addition of Stop Solution. The signal was less stable in samples of purified LDH enzyme diluted in PBS + 1mg/ml BSA. Additional studies indicate the fluorescence begins to drop after 1-2 hours for samples in PBS +1mg/ml BSA. L929 cells were cultured in serum supplemented medium and plates were stored at room temperature protected from light after addition of Stop Solution.

Comparison of Filter Sets and Plate Readers for CytoTox-ONE™ Assay

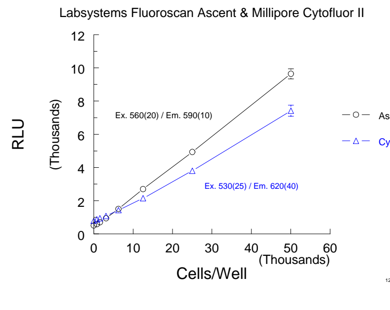
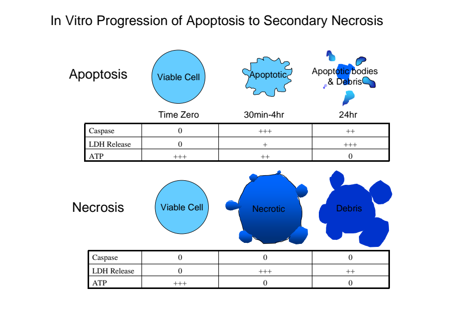


Fig. 8. A comparison of two fluorescent plate readers with different filter sets demonstrates the ability to record fluorescence at less than optimal excitation and emission wavelengths. The recommended wavelengths to monitor the assay are 560nm excitation & 590nm emission. However, the peaks are broad, and one can excite from 530-570nm and measure emission from 580-620nm. The CytoFluor II instrument captured fluorescence using a 530(25)nm excitation and 620(40)nm emission filiter pair. The Labsystems Fluroscan Ascent reader was equipped with the optimal 560(20)/590(10)nm filters.

Duration of Exposure and Concentration of Test Compound May Affect Cytotoxicity Assay Data

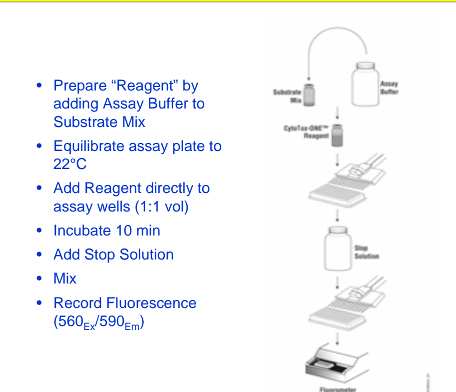


Apoptosis	Time Zero	30min-4hr	24hr
Caspase	0	+++	++
LDH Release	0	+	+++
ATP	+++	++	0

Necrosis	Time Zero	30min-4hr	24hr
Caspase	0	0	0
LDH Release	0	+++	++
ATP	+++	0	0

Fig. 11. Design of cytotoxicity assay protocols affect results. Using *in vitro* model systems, apoptotic cells eventually undergo secondary necrosis and release their contents (e.g. LDH) into the culture medium. High doses of compounds known to induce apoptosis may induce immediate necrosis without induction of caspase activity. Testing multiple drug concentrations, using multiple cytotoxicity markers, and recording observations at various times, may help avoid misinterpretation of screening data.

Homogeneous Protocol to Measure Cytotoxicity



- Prepare "Reagent" by adding Assay Buffer to Substrate Mix
- Equilibrate assay plate to 22°C
- Add Reagent directly to assay wells (1:1 vol)
- Incubate 10 min
- Add Stop Solution
- Mix
- Record Fluorescence ($560_{Ex}/590_{Em}$)

Comparison of CytoTox-ONE™ and CytoTox 96® Assays HepG2 Cells Exposed to Tamoxifen 4hr

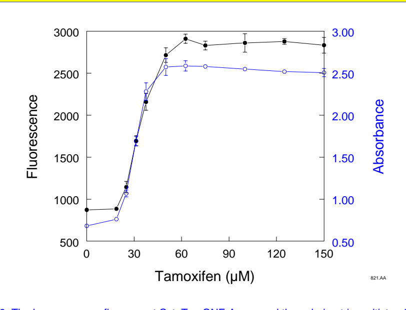


Fig. 3. The homogeneous fluorescent CytoTox-ONE Assay and the colorimetric multistep CytoTox 96® Assay generate similar IC_{50} values. HepG2 cells (15,000/well) were cultured in MEM + 10% FBS, 1mM sodium pyruvate and 0.1mM non-essential amino acids. Cells were exposed to various concentrations of tamoxifen for 4 hr. The standard CytoTox-ONE Assay protocol was followed. The colorimetric multistep CytoTox 96® Assay was done by transferring 50µl of supernatant from the assay plate into a separate enzymatic reaction plate containing CytoTox 96® Reagent. After 30min incubation at 22°C, absorbance 490nm was recorded for the colorimetric assay.

Culture Medium Supplemented with Pyruvate Reduces Rate of LDH Detection Reaction

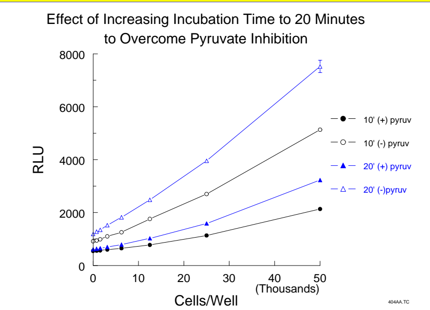


Fig. 6. Pyruvate supplementation of culture medium resulted in inhibition of the rate of CytoTox-ONE™ Assay reactions. Sensitivity can be increased by doubling the incubation time to 20 min to compensate for pyruvate inhibition of LDH. Serial dilutions of HepG2 cells were prepared in MEM +10% FBS, with and without 1mM sodium pyruvate. Cells were lysed and fluorescence was determined using the CytoTox-ONE™ Assay. Sensitivity was somewhat diminished when using medium supplemented with pyruvate.

Similar IC_{50} Values from CytoTox-ONE™ (Cytotoxicity) and CellTiter-Glo™ (Viability) Assays

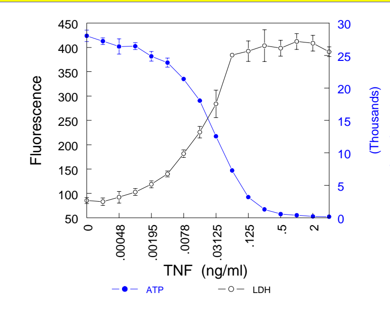


Fig. 9. IC_{50} values were determined to be 0.027 ng/ml for ATP determination and 0.018 for LDH. L929 cells (2000 cells/well in 15µl F12/DME +10% HS) were cultured in a 384 well plates for 24hr at 37°C, 5% CO_2. TNFα (n=4, 10µl additions) in the presence of actinomycin D (1µg/ml final) was added and incubated for 20hr. CytoTox-ONE™ Reagent (25µl/well) was added, the plate shaken for 30 sec, incubated for 10 min at 22°C, then 12.5µl/well of Stop Solution were added. To determine ATP concentration, the homogeneous CellTiter-Glo™ Assay was done in parallel using the recommended procedure for 384 well plates.

Summary

- CytoTox-ONE™ Assay is a homogeneous method to measure the number of "dead" cells in culture by detecting release of LDH through compromised cell membranes.
- The fluorescent signal is directly proportional to the number of "dead" cells in cultures containing mixtures of live and dead cells.
- The long half-life of released LDH provides advantages over methods for measuring other released components.
- The homogeneous fluorescent method is directly comparable to the non-homogeneous colorimetric LDH assay.
- The reconstituted CytoTox-ONE™ Reagent is stable upon storage making it convenient for robotic HTS applications.
- The fluorescent signal from the CytoTox-ONE™ Assay of cells in culture is stable for 48 hours.
- LDH-release cytotoxicity assays (CytoTox-ONE™ Assay) correlate inversely with ATP content viability assays (CellTiter-Glo™ Assay).
- Multiplexing: Samples can be transferred to a separate CytoTox-ONE™ Assay plate leaving the original culture plate unaltered and available for any other assay (e.g. cell viability, apoptosis, reporter gene assay, kinase assay, cell imaging etc.)
- For technical information, see: www.promega.com/tbs/tb306/tb306.html