CONTENTS

Promega

I. Introduction

Choosing a cell viability or cytotoxicity assay from among the many different options available can be a challenging task. Picking the best assay format to suit particular needs requires an understanding of what each assay is measuring as an endpoint, of how the measurement correlates with cell viability, and of what the limitations of the assay chemistries are. Here we provide recommendations for characterizing a model assay system and some of the factors to consider when choosing cell-based assays for manual or automated systems.

A. Establishing an In Vitro Model System

The species of origin and cell types used in cytotoxicity studies are often dictated by specific project goals or the drug target that is being investigated. Regardless of the model system chosen, establishing a consistent and reproducible procedure for setting up assay plates is important. The number of cells per well and the equilibration period prior to the assay may affect cellular physiology. Maintenance and handling of stock cultures at each step of the manufacturing process should be standardized and validated for consistency. Assay responsiveness to test compounds can be influenced by many subtle factors including culture medium surface-to-volume ratio, gas exchange, evaporation of liquids and edge effects. These factors are especially important considerations when attempting to scaleup assay throughput.

B. Choosing an Endpoint to Measure

One of the first things to decide before choosing an assay is exactly what information you want to measure at the end of a treatment period. Assays are available to measure a variety of different markers that indicate the number of dead cells (cytotoxicity assay), the number of live cells (viability assay), the total number of cells or the mechanism of cell death (e.g., apoptosis). Table 4.1 compares Promega homogeneous cell-based assays and lists the measured parameters, sensitivity of detection, incubation time and detection method for each assay.

A basic understanding of the changes that occur during different mechanisms of cell death will help you decide which endpoint to choose for a cytotoxicity assay (Riss and Moravec, 2004). Figure 4.1 shows a simplified example illustrating chronological changes occurring during apoptosis and necrosis and the results that would be expected from using the assays listed in Table 4.1 to measure different markers.

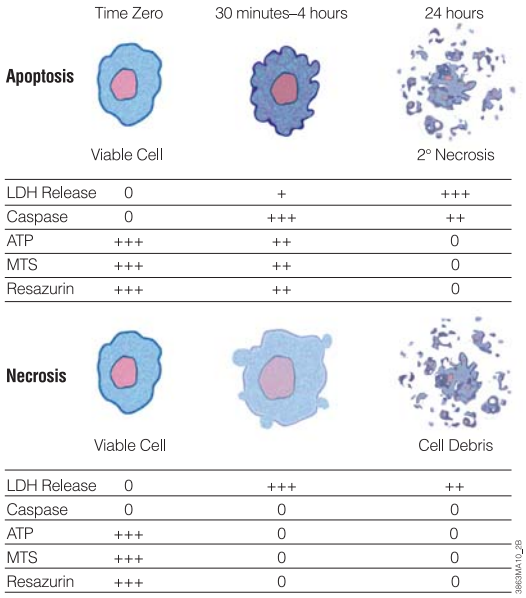

Figure 4.1. Mechanisms of cell death can be determined by measuring different markers of cell viability and apoptosis in vitro.

Cultured cells that are undergoing apoptosis in vitro eventually undergo secondary necrosis. After extended incubation, apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium. Markers of apoptosis such as caspase activity may be present only transiently. Therefore, to determine if apoptosis is the primary mechanism of cell death, understanding the kinetics of the cell death process in your model system is critical.

Cells undergoing necrosis typically undergo rapid swelling, lose membrane integrity, shut down metabolism and release their cytoplasmic contents into the surrounding culture medium. Cells undergoing rapid necrosis in vitro do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers. [For additional information about mechanisms of cell death, please visit the Apoptosis Chapter of this *Protocols and Applications Guide*.]

If the information sought is simply whether there is a difference between "no treatment" negative controls and "toxin treatment" of experimental wells, the choice between measuring the number of viable cells or the number of dead cells may be irrelevant. However, if more detailed information on the mechanism of cell death is being sought, the duration of exposure to toxin, the concentration of the test compound, and the choice of the assay endpoint become critical (Riss and Moravec, 2004).

C. Characterizing Assay Responsiveness

Protocols used to measure cytotoxicity in vitro differ widely. Often assay plates are set up containing cells and allowed to equilibrate for a predetermined period before adding test compounds. Alternatively, cells may be added

Table 4.1. Comparison of Promega Cell Viability, Cytotoxicity and Apoptosis Assays

Characteristic	CellTiter-Glo® Luminescent Cell Viability Assay	BacTiter-Glo® Microbial Cell Viability Assay	CellTiter-Blue® Cell Viability Assay	CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay
Incubation	10 minutes	5 minutes	1–4 hours	1–4 hours
Parameter measured	ATP	ATP	resazurin reduction	MTS reduction
Sensitivity: 96-well/384-well	50 cells/15 cells (also 1536-well format)	~40 cells/N.D.	390 cells/50 cells	800 cells/200 cells
Sample Type	suspension or adherent cells	bacteria, yeast	suspension or adherent cells	suspension or adherent cells
Detection	luminescent	luminescent	fluorometric or colorimetric	colorimetric

Characteristic	Apo-ONE® Homogeneous Caspase-3/7 Assay	Caspase-Glo® 3/7 Assay	Caspase-Glo® 8 or 9 Assays
Incubation	1–18 hours	30 minutes–2 hours	30 minutes–2 hours
Parameter measured	effector caspase activity	effector caspase activity	initiator caspase activity
Sensitivity: 96-well/384-well	Several hundred cells in a population	20 cells/20 cells	20 cells/20 cells
Sample Type	culture cells or purified enzyme	culture cells or purified enzyme	culture cells or purified enzyme
Detection	fluorometric	luminescent	luminescent

Characteristic	CytoTox-ONE™ Membrane Integrity Assay	MultiTox-Fluor Multiplex Cytotoxicity Assay	CytoTox-Fluor™ Cytotoxicity Assay	CytoTox 96® Non-Radioactive Cytotoxicity Assay
Incubation	10 minutes	30 minutes	30 minutes	30 minutes
Parameter measured	LDH release	live- and dead-cell protease activity	dead-cell protease activity	LDH Release
Sensitivity: 96-well/384-well	800 cells/200 cells	several hundred cells or cell equivalents (also in 1536-well format)	several hundred cell equivalents	several hundred cells or cell equivalents
Sample Type	suspension or adherent cells	suspension or adherent cells	suspension or adherent cells	suspension or adherent cells
Detection	fluorometric	fluorometric	fluorometric	colorimetric

directly to plates that already contain test compounds. The duration of exposure to the toxin may vary from less than an hour to several days, depending on specific project goals. Brief periods of exposure may be used to determine if test compounds cause an immediate necrotic insult to cells, whereas exposure for several days is commonly used to determine if test compounds inhibit cell proliferation. Cell viability or cytotoxicity measurements usually are determined at the end of the exposure period. Assays that require only a few minutes to generate a measurable signal (e.g., ATP quantitation or LDH-release assays) provide information representing a snapshot in time and have an advantage over assays that may require several hours of incubation to develop a signal (e.g., MTS or resazurin). In addition to being more convenient, rapid assays reduce the chance of artifacts caused by interaction of the test compound with assay chemistry.

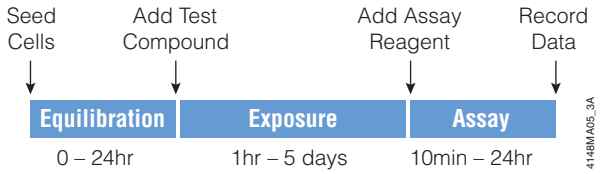

Figure 4.2. Generalized scheme representing an in vitro cytotoxicity assay protocol.

In vitro cultured cells exist as a heterogeneous population. When populations of cells are exposed to test compounds, they do not all respond simultaneously. Cells exposed to toxin may respond over the course of several hours or days, depending on many factors, including the mechanism of cell death, the concentration of the toxin and the duration of exposure. As a result of culture heterogeneity, the data from most plate-based assay formats represent an average of the signal from the population of cells.

D. Determining Dose and Duration of Exposure

Characterizing assay responsiveness for each in vitro model system is important, especially when trying to distinguish between different mechanisms of cell death (Riss and Moravec, 2004). Initial characterization experiments should include a determination of the appropriate assay window using an established positive control.

Figures 4.3 and 4.4 show the results of two experiments to determine the kinetics of cell death caused by different concentrations of tamoxifen in HepG2 cells. The two experiments measured different endpoints: ATP as an indicator of viable cells and caspase activity as a marker for apoptotic cells.

The ATP data in Figure 4.3 indicate that high concentrations of tamoxifen are toxic after a 30-minute exposure. The longer the duration of tamoxifen exposure the lower the IC_{50} value or dose required to "kill" half of the cells, suggesting the occurrence of a cumulative cytotoxic effect. Both the concentration of toxin and the duration of exposure contribute to the cytotoxic effect. To illustrate the importance of taking measurements after an appropriate duration of exposure to test compound, notice that the ATP assay indicates that 30µM tamoxifen is not toxic at short incubation times but is 100% toxic after 24 hours of exposure. Choosing the appropriate incubation period will affect results.

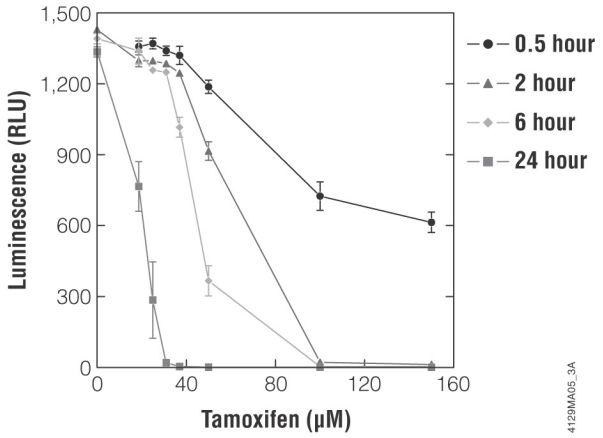

Figure 4.3. Characterization of the toxic effects of tamoxifen on HepG2 cells using the CellTiter-Glo® Luminescent Cell Viability Assay to measure ATP as an indication of cell viability.

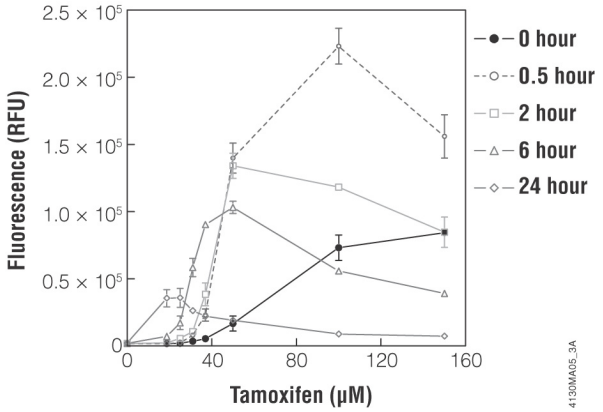

Figure 4.4. Characterization of the effects of tamoxifen on HepG2 cells using the Apo-ONE® Homogeneous Caspase-3/7 Assay to measure caspase-3/7 activity as a marker of apoptosis.

The appearance of some apoptosis markers is transient and may only be detectable within a limited window of time. The data from the caspase assay in Figure 4.4 illustrate the transient nature of caspase activity in cells undergoing apoptosis. The total amount of caspase activity measured after a 24-hour exposure to tamoxifen is only a fraction of earlier time points. There is a similar trend of shifting to lower IC_{50} values after increased exposure time. The combined ATP and caspase data may suggest that, at early time points with intermediate concentrations of tamoxifen, the cells are undergoing apoptosis; but after a 24-hour exposure most of the population of cells are in a state of secondary necrosis.

E. Homogeneous Assays for Multiwell Formats and Automated Screening

Promega produces a complete portfolio of homogeneous assays (assays that can be performed in your cell culture plates) that are designed to meet a variety of experimental requirements. The general protocol for these "homogeneous" assays is "add, mix and measure." Some of these homogeneous assay systems require combining components to create the "reagent," and some protocols require incubation or agitation steps, but none require removing buffer or medium from assay wells. The available homogeneous assay systems include assays designed to measure cell viability, cytotoxicity and apoptosis. Promega also offers some non-homogeneous cell viability assays.

F. Additional Factors to Consider When Choosing a Cell Viability Assay

Among the many factors to consider when choosing a cell-based assay, the primary concern for many researchers is the **ease of use.** Homogeneous assays do not require removal of culture medium, cell washes or centrifugation steps. When choosing an assay, the time required for reagent preparation and the total length of time necessary to develop a signal from the assay chemistry should be considered. The stability of the absorbance, fluorescence or luminescence signal is another important factor that provides convenience and flexibility in recording data and minimizes differences when processing large batches of plates.

Another factor to consider when selecting an assay is **sensitivity of detection**. Detection sensitivity will vary with cell type if you choose to measure a metabolic marker, such as ATP level or MTS tetrazolium reduction. The signal-to-background ratios of some assays may be improved by increasing incubation time. The sensitivity not only depends upon the parameter being measured but also on other parameters of the model system such as the plate format and number of cells used per well. Cytotoxicity assays that are designed to detect a change in viability in a population of 10,000 cells may not require the most sensitive assay technology. For example, a tetrazolium assay should easily detect the difference between 10,000 and 8,000 viable cells. On the other hand, assay model systems that use low cell numbers in a high-density multiwell plate format may require maximum sensitivity of detection such as that achieved with the luminescent ATP assay technology.

For researchers using automated screening systems, the **reagent stability** and compatibility with robotic components is often a concern. The assay reagents must be stable at ambient temperature for an adequate period of time to complete dispensing into several plates. In addition, the signal generated by the assay should also be stable for extended periods of time to allow flexibility for recording data. For example, the luminescent signal from the ATP assay has a half-life of about 5 hours, providing adequate flexibility. With other formats such as the MTS tetrazolium assay or the LDH release assay, the signal can be stabilized by the addition of a detergent-containing stop solution.

In some cases the choice of assay may be dictated by the **availability of instrumentation** to detect absorbance, fluorescence or luminescence. The Promega portfolio of products contains an optional detection format for each of the three major classes of cell-based assays (viability, cytotoxicity or apoptosis). In addition, results from some assays such as the ATP assay can be recorded with more than one type of instrument (luminometer, fluorometer or CCD camera).

Cost is an important consideration for every researcher; however, many factors that influence the **total cost of running an assay** are often overlooked. All of the assays described above are homogeneous and as such are more efficient than multistep assays. For example, even though the reagent cost of an ATP assay may be higher than other assays, the speed (time savings), sensitivity (cell sample savings) and accuracy may outweigh the initial cost. Assays with good detection sensitivity that are easier to scale down to 384- or 1536-well formats may result in savings of cell culture reagents and enable testing of very small quantities of expensive or rare test compounds.

The ability to gather more than one set of data from the same sample (i.e., multiplexing) also may contribute to saving time and effort. **Multiplexing** more than one assay in the same culture well can provide internal controls and eliminate the need to repeat work. For instance, the LDH-release assay is an example of an assay that can be multiplexed. The LDH-release assay offers the opportunity to gather cytotoxicity data from small aliquots of culture supernatant that can be removed to a separate assay plate, thus leaving the original assay plate available for any other assay such as gene reporter analysis, image analysis, etc. Several of our homogeneous apoptosis and viability assays can be multiplexed without transferring media, allowing researchers to assay multiple parameters in the same sample well.

Reproducibility of data is an important consideration when choosing a commercial assay. However, for most cell-based assays, the variation among replicate samples is more likely to be caused by the cells rather than the assay chemistry. Variations during plating of cells can be magnified by using cells lines that tend to form clumps rather than a suspension of individual cells. Extended incubation periods and edge effects in plates may also lead to decreased reproducibility among replicates and less desirable Z'-factor values.

Promega Publications
Timing Your Apoptosis Assays

II. Cell Viability Assays that Measure ATP

A. CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay is a homogeneous method to determine the number of viable cells in culture. Detection is based on using the luciferase

reaction to measure the amount of ATP from viable cells. The amount of ATP in cells correlates with cell viability. Within minutes after a loss of membrane integrity, cells lose the ability to synthesize ATP, and endogenous ATPases destroy any remaining ATP; thus the levels of ATP fall precipitously. The CellTiter-Glo® Reagent does three things upon addition to cells. It lyses cell membranes to release ATP; it inhibits endogenous ATPases, and it provides luciferin, luciferase and other reagents necessary to measure ATP using a bioluminescent reaction.

The unique properties of a proprietary stable luciferase mutant enabled a robust, single-addition reagent. The "glow-type" signal can be recorded with a luminometer, CCD camera or modified fluorometer and generally has a half-life of five hours, providing a consistent signal across large batches of plates. The CellTiter-Glo® Assay is extremely sensitive and can detect as few as 10 cells. The luminescent signal can be detected as soon as 10 minutes after adding reagent, or several hours later, providing flexibility for batch processing of plates.

Materials Required:

- CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572, G7573) and protocol #TB288
- opaque-walled multiwell plates adequate for cell culture
- multichannel pipette or automated pipetting station
- plate shaker, for mixing multiwell plates
- luminometer (e.g., GloMax™ 96 Microplate Luminometer (Cat.# E6501) or CCD imager capable of reading multiwell plates
- ATP (for use in generating a standard curve)

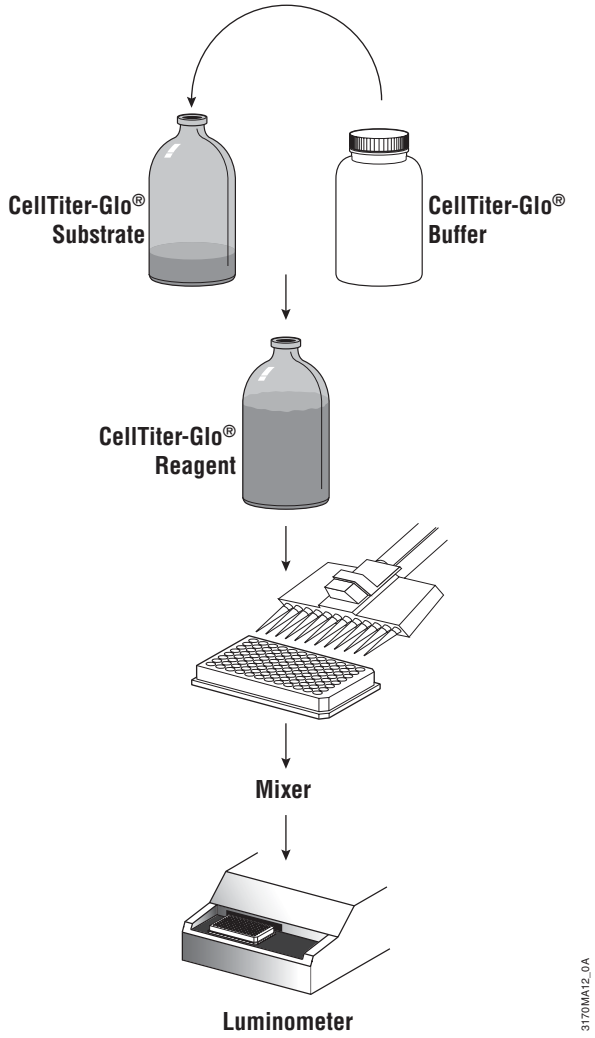

Figure 4.5. Schematic diagram of CellTiter-Glo® Luminescent Cell Viability Assay protocol. For a detailed protocol and considerations for performing this assay, see Technical Bulletin #TB288.

Additional Considerations for Performing the CellTiter-Glo® Luminescent Cell Viability Assay

Temperature: The intensity and rate of decay of the luminescent signal from the CellTiter-Glo® Assay depends on the rate of the luciferase reaction. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature before performing the assay. Transferring eukaryotic cells from 37°C to room temperature has little effect on the ATP content (Lundin *et al*. 1986). We have demonstrated that removing cultured cells from a 37°C incubator and allowing them to equilibrate to 22°C for 1–2 hours has little effect on the ATP content.

For batch-mode processing of multiple assay plates, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer equilibration than plates arranged in a single layer. Insufficient

equilibration may result in a temperature gradient effect between the wells in the center and on the edge of the plates. The temperature gradient pattern may depend on the position of the plate in the stack.

Chemicals: Differences in luminescence intensity have been observed using different types of culture media and sera. The presence of phenol red in culture medium should have little impact on luminescence output. Assay of 0.1µM ATP in RPMI medium without phenol red showed ~5% increase in relative light units (RLU) compared to RPMI containing the standard concentration of phenol red, whereas RPMI medium containing 2X the normal concentration of phenol red showed a ~2% decrease in RLU. Solvents used for the various test compounds may interfere with the luciferase reaction and thus affect the light output from the assay. Interference with the luciferase reaction can be determined by assaying a parallel set of control wells containing medium without cells. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and only minimally affects light output.

Plate Recommendations: We recommend using opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms to allow microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape can be used to decrease luminescence loss and cross-talk.

Cellular ATP Content: Values reported for the ATP level in cells vary considerably (Lundin *et al*. 1986; Kangas *et al*. 1984; Stanley, 1986; Beckers *et al*. 1986; Andreotti *et al*. 1995). Factors that affect the ATP content of cells may affect the relationship between cell number and luminescence. Anchorage-dependent cells that undergo contact inhibition at high densities may show a change in ATP content per cell at high densities, resulting in a nonlinear relationship between cell number and luminescence. Factors that affect the cytoplasmic volume or physiology of cells also can affect ATP content. For example, depletion of oxygen is one factor known to cause a rapid decrease in ATP (Crouch *et al*. 1993).

Mixing: Optimum assay performance is achieved when the CellTiter-Glo® Reagent is completely mixed with the sample of cultured cells. Suspension cell lines (e.g., Jurkat cells) generally require less mixing to achieve lysis and extraction of ATP than adherent cells (e.g., L929 cells). Several additional parameters related to reagent mixing include: the force of delivery of CellTiter-Glo® Reagent, the sample volume and the dimensions of the well. All of these factors may affect assay performance. The degree of mixing required may be affected by the method used for adding the CellTiter-Glo® Reagent to the assay plates. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Complete reagent mixing in 96-well plates should be achieved using orbital plate shaking devices, which are built into many luminometers, and shaking for the recommended 2 minutes. Special electromagnetic shaking devices using a radius smaller than the diameter of the well may be required when using 384-well plates. The depth of the medium and the geometry of the multiwell plates may also affect mixing efficiency.

Additional Resources for CellTiter-Glo® Luminescent Cell Viability Assay

Technical Bulletins and Manuals

TB288 *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin*

EP014 *Automated CellTiter-Glo® Luminescent Cell Viability Assay Protocol*

Promega Publications

Selecting cell-based assays for drug-discovery screening

Multiplexing homogeneous cell-based assays

Choosing the right cell-based assay for your research

CellTiter-Glo® Luminescent Cell Viability Assay for cytoxicity and cell proliferation studies

Online Tools

Cell Viability Assistant

Citations

Nguyen, D.G. *et al.* (2006) "UnPAKing" Human Immunodeficiency Virus (HIV) replication: Using small interfering RNA screening to identify novel cofactors and elucidate the role of Group I PAKs in HIV infection. *J. Virol.* **80**, 130–7.

The CellTiter-Glo® Luminescent Cell Viability Assay was used to assess viability of HeLaCD4βgal or U373-Magi-CCR5E cells transfected with siRNAs that targeted potential proviral host factors for HIV infection.

PubMed Number: 16352537

Boutros, M. *et al.* (2004) Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. *Science* **303**, 832–5.

This paper decribes use of RNA interference (RNAi) to screen the genome of *Drosophila melanogaster* for genes affecting cell growth and viability. The CellTiter-Glo® Luminescent Cell Viability Assay and a Molecular Dynamics Analyst HT were used. The authors report finding 438 target genes that affected cell growth or viability.

PubMed Number: 14764878

B. BacTiter-Glo™ Microbial Cell Viability Assay

The BacTiter-Glo™ Microbial Cell Viability Assay is based on the same assay principles and chemistries as the CellTiter-Glo® Assay. However, the buffer supports bacterial cell lysis of Gram+ and Gram– bacteria and yeast. Figure 4.6 provides a basic outline of the BacTiter-Glo™ Assay procedure. The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal in an "add, mix and measure" format. This assay can measure ATP from as few as ten bacterial cells from some species and is a powerful tool for determining growth

curves of slow-growing microorganisms (Figure 4.7), screening for antimicrobial compounds (Figure 4.8) and evaluating antimicrobial compounds (Figure 4.9).

Materials Required:

- BacTiter-Glo™ Luminescent Cell Viability Assay (Cat.# G8230, G8231, G8232, G8233) and protocol #TB337
- opaque-walled multiwell plates
- multichannel pipette or automated pipetting station
- plate shaker, for mixing multiwell plates
- luminometer (e.g., GloMax™ 96 Microplate Luminometer (Cat.# E6501) or CCD imager capable of reading multiwell plates
- ATP (for generating a standard curve; Cat.# P1132)

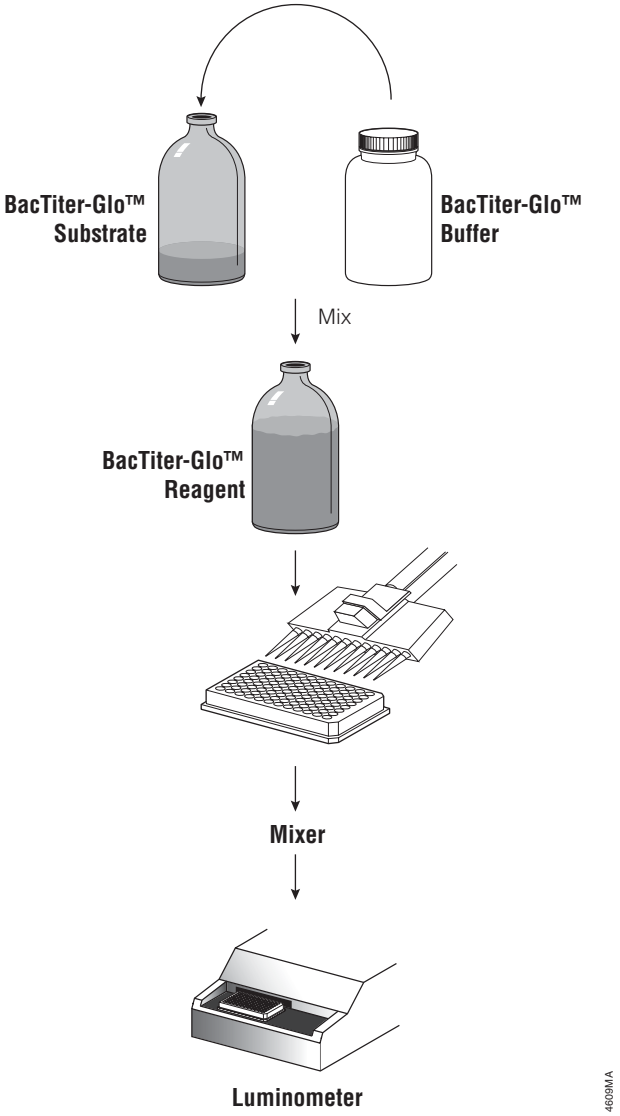

Figure 4.6. Schematic diagram of BacTiter-Glo™ Assay protocol. For a detailed protocol and considerations for performing this assay, see Technical Bulletin #TB337. The assay is suitable for single-tube or multiwell-plate formats.

Additional Considerations for Performing the BacTiter-Glo™ Microbial Cell Viability Assay

Temperature: The intensity and rate of decay of the luminescent signal from the BacTiter-Glo™ Assay depend on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to room temperature before performing the assay. Insufficient equilibration may create a temperature gradient effect between the wells in the center and on the edge of the plates.

Microbial Growth Medium: Growth medium is another factor that can contribute to the background luminescence and affect the luciferase reaction in terms of signal level and signal stability. We have used MH II Broth (cation-adjusted Mueller Hinton Broth; Becton, Dickinson and Company Cat.# 297963) for all our experiments unless otherwise mentioned. It supports growth for most commonly encountered aerobic and facultative anaerobic bacteria and is selected for use in food testing and antimicrobial susceptibility testing by Food and Drug Administration and National Committee for Clinical Laboratory Standards (NCCLS) (Association of Official Analytical Chemists, 1995; NCCLS, 2000). MH II Broth has low luminescence background and good batch-to-batch reproducibility.

Chemicals: The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for the various compounds tested for their antimicrobial activities may interfere with the luciferase reaction and thus the light output from the assay. Interference with the luciferase reaction can be detected by assaying a parallel set of control wells containing medium without compound. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations up to 2% in the assay and has less than 5% loss of light output.

Plate and Tube Recommendations: The BacTiter-Glo™ Assay is suitable for multiwell-plate or single-tube formats. We recommend standard opaque-walled multiwell plates suitable for luminescence measurements. Opaque-walled plates with clear bottoms to allow microscopic visualization of cells also may be used; however, these plates will have diminished signal intensity and greater cross-talk between wells. Opaque white tape can be used to reduce luminescence loss and cross-talk. For single-tube assays, the standard tube accompanying the luminometer should be suitable.

Cellular ATP Content: Different bacteria have different amounts of ATP per cell, and values reported for the ATP level in cells vary considerably (Stanley, 1986; Hattori *et al*. 2003). Factors that affect the ATP content of cells such as

growth phase, medium, and presence of metabolic inhibitors, may affect the relationship between cell number and luminescence (Stanley, 1986).

Mixing: Optimum assay performance is achieved when the BacTiter-Glo™ Reagent is completely mixed with the sample of cultured cells. For all of the bacteria we tested, maximum luminescent signals were observed after efficiently mixing and incubating for 1–5 minutes. However, complete extraction of ATP from certain bacteria, yeast or fungi may take longer. Automated pipetting devices using a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Ensure complete reagent mixing in 96-well plates by using orbital plate shaking devices built into many luminometers. We recommend considering these factors when performing the assay and determining whether a mixing step and/or longer incubation is necessary.

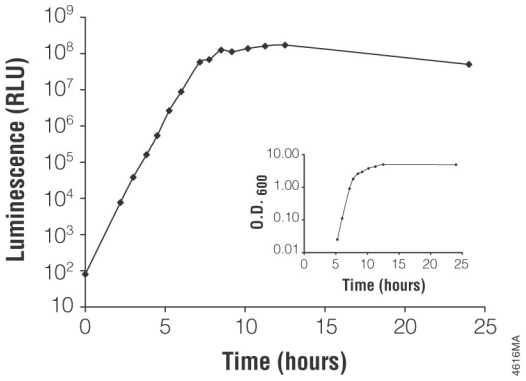

Figure 4.7. Evaluating bacterial growth using the BacTiter-Glo™ Assay. *E. coli* ATCC 25922 strain was grown in Mueller Hinton II (MH II) broth (B.D. Cat.# 297963) at 37°C overnight. The overnight culture was diluted 1:10^6 in 50ml of fresh MH II broth and incubated at 37°C with shaking at 250rpm. Samples were taken at various time points, and the BacTiter-Glo™ Assay was performed according to the protocol described in Technical Bulletin #TB337. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Cat.# E6501). Optical density was measured at 600nm ($O.D._{600}$) using a Beckman DU650 spectrophotometer. Diluted samples were used when readings of relative light units (RLU) and O.D. exceeded 10^8 and 1, respectively.

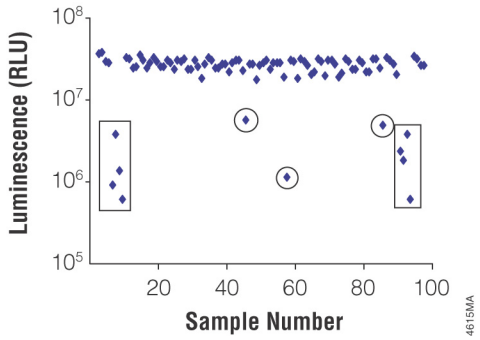

Figure 4.8. Screening for antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain was grown in Mueller Hinton II (MH II) Broth (BD Cat.# 297963) at 37°C overnight. The overnight culture was diluted 100-fold in fresh MH II Broth and used as inoculum for the antimicrobial screen. Working stocks (50X) of LOPAC compounds and standard antibiotics were prepared in DMSO. Each well of the 96-well plate contained 245µl of the inoculum and 5µl of the 50X working stock. The multiwell plate was incubated at 37°C for 5 hours. One hundred microliters of the culture was taken from each well, and the BacTiter-Glo™ Assay was performed according to the protocol described in Technical Bulletin #TB337. Luminescence was measured using a GloMax™ 96 Microplate Luminometer (Cat.# E6501). The samples and concentrations are: wells 1–4 and 93–96, negative control of 2% DMSO; wells 5–8 and 89–92, positive controls of 32µg/ml standard antibiotics tetracycline, ampicillin, gentamicin, chloramphenicol, oxacillin, kanamycin, piperacillin and erythromycin; wells 9–88, LOPAC compounds at 10µM.

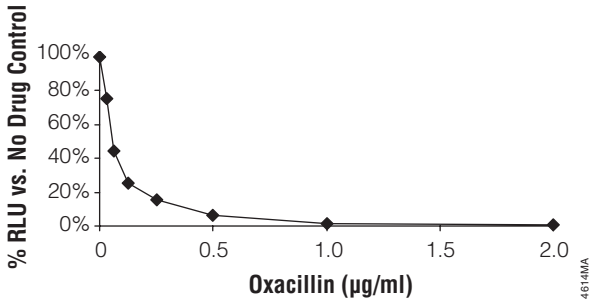

Figure 4.9. Evaluating antimicrobial compounds using the BacTiter-Glo™ Assay. *S. aureus* ATCC 25923 strain and oxacillin were prepared as described in Figure 4.8 and incubated at 37°C; the assay was performed after 19 hours of incubation as recommended for MIC determination by NCCLS. The percentage of relative light units (RLU) compared to the no-oxacillin control is shown. Luminescence was recorded on a GloMax™ 96 Microplate Luminometer (Cat.# E6501).

Additional Resources for BacTiter-Glo™ Microbial Cell Viability Assay

Technical Bulletins and Manuals

TB337 *BacTiter-Glo™ Microbial Cell Viability Assay Technical Bulletin*

Promega Publications

Determining microbial viability using a homogeneous luminescent assay

Quantitate microbial cells using a rapid and sensitive ATP-based luminescent assay

III. Cell Viability Assays that Measure Metabolic Capacity

A. CellTiter-Blue® Cell Viability Assay (resazurin)

The CellTiter-Blue® Cell Viability Assay uses an optimized reagent containing resazurin. The homogeneous procedure involves adding the reagent directly to cells in culture at a recommended ratio of 20µl of reagent to 100µl of culture medium. The assay plates are incubated at 37°C for 1–4 hours to allow viable cells to convert resazurin to the fluorescent resorufin product. The conversion of resazurin to fluorescent resorufin is proportional to the number of metabolically active, viable cells present in a population (Figure 4.10). The signal is recorded using a standard multiwell fluorometer. Because different cell types have different abilities to reduce resazurin, optimizing the length of incubation with the CellTiter-Blue® Reagent can improve assay sensitivity for a given model system. The detection sensitivity is intermediate between the ATP assay and the MTS reduction assay.

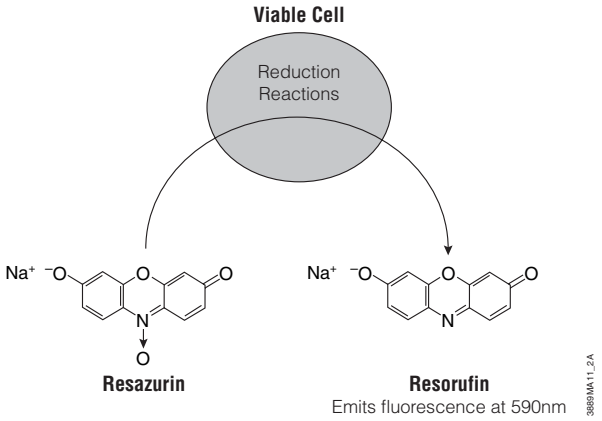

Figure 4.10. Conversion of resazurin to resorufin by viable cells results in a fluorescent product. The fluorescence produced is proportional to the number of viable cells.

The CellTiter-Blue® Assay is a simple and inexpensive procedure that is amenable to multiplexing applications with other assays to collect a variety of data (Figures 4.11 and 4.12). The incubation period is flexible, and the data can be collected using either fluorescence or absorbance, though fluorescence is preferred because of superior sensitivity. The assay provides good Z′-factor values in high-throughput screening situations and is amenable to automation.

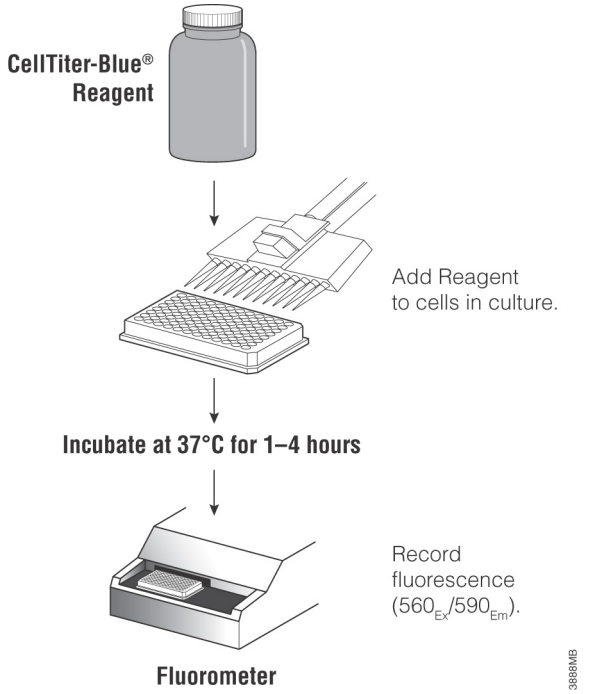

Figure 4.11. Schematic outlining the CellTiter-Blue® Assay protocol. Multiwell plates that are compatible with fluorescent plate readers are prepared with cells and compounds to be tested. CellTiter-Blue® Reagent is added to each well and incubated at 37°C to allow cells to convert resazurin to resorufin. The fluorescent signal is read using a fluorescence plate reader.

Materials Required:

- CellTiter-Blue® Cell Viability Assay (Cat.# G8080, G8081, G8082) and protocol #TB317
- multichannel pipettor
- fluorescence reader with excitation 530–570nm and emission 580–620nm filter pair
- absorbance reader with 570nm and 600nm filters (optional)
- 96-well plates compatible with a fluorescence plate reader

General Considerations for the CellTiter-Blue® Cell Viability Assay

Incubation Time: The ability of different cell types to reduce resazurin to resorufin varies depending on the metabolic capacity of the cell line and the length of incubation with the CellTiter-Blue® Reagent. For most applications a 1- to 4-hour incubation is adequate. For optimizing screening assays, the number of cells/well and the length of the incubation period should be empirically determined. A more detailed discussion of incubation time is available in Technical Bulletin #TB317.

Volume of Reagent Used: The recommended volume of CellTiter-Blue® Reagent is 20µl of reagent to each 100µl of medium in a 96-well format or 5µl of reagent to each 25µl of culture medium in a 384-well format. This ratio may be adjusted for optimal performance, depending on the cell type, incubation time and linear range desired.

Site of Resazurin Reduction: Resazurin is reduced to resorufin inside living cells (O'Brien *et al*. 2000). Resazurin can penetrate cells, where it becomes reduced to the fluorescent product, resorufin, probably as the result of the action of several different redox enzymes. The fluorescent resorufin dye can diffuse from cells and back into the surrounding medium. Culture medium harvested from rapidly growing cells does not reduce resazurin (O'Brien *et al*. 2000). An analysis of the ability of various hepatic subcellular fractions suggests that resazurin can be reduced by mitochondrial, cytosolic and microsomal enzymes (Gonzalez and Tarloff, 2001).

Optical Properties of Resazurin and Resorufin: Both the light absorbance and fluorescence properties of the CellTiter-Blue® Reagent are changed by cellular reduction of resazurin to resorufin; thus either absorbance or fluorescence measurements can be used to monitor results. We recommend measuring fluorescence because it is more sensitive than absorbance and requires fewer calculations to account for the overlapping absorbance spectra of resazurin and resorufin. More details about making fluorescence and absorbance measurements are provided in Technical Bulletin #TB317.

Background Fluorescence and Light Sensitivity of Resazurin: The resazurin dye (blue) in the CellTiter-Blue® Reagent and the resorufin product produced in the assay (pink) are light-sensitive. Prolonged exposure of the CellTiter-Blue® Reagent to light will result in increased background fluorescence and decreased sensitivity. Background fluorescence can be corrected by including control wells on each plate to measure the fluorescence from serum-supplemented culture medium in the absence of cells. There may be an increase in background fluorescence in wells without cells after several hours of incubation.

Multiplexing with Other Assays: Because CellTiter-Blue® Reagent is relatively non-destructive to cells during short-term exposure, it is possible to use the same culture wells to do more than one type of assay. An example showing the measurement of caspase activity using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7792) is shown in Figure 4.12. A protocol for multiplexing the CellTiter-Blue® Assay and the Apo-ONE® Caspase-3/7 Assay is provided in chapter 3 of this *Protocols and Applications Guide*.

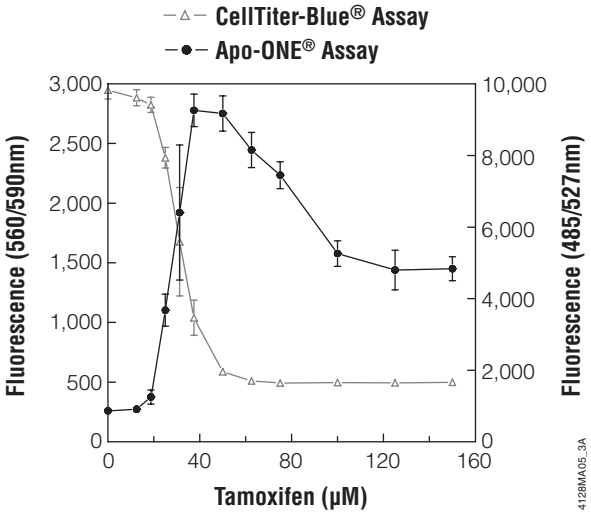

Figure 4.12. Multiplexing the CellTiter-Blue® Assay with the Apo-ONE® Homogeneous Caspase-3/7 Assay. HepG2 cells (10,000cells/100µl cultured overnight) were treated with various concentrations of tamoxifen for 5 hours. Viability was determined by adding CellTiter-Blue® Reagent (20µl/well) to each well after 3.5 hours of drug treatment and incubating for 1 hour before recording fluorescence ($560_{Ex}/590_{Em}$). Caspase activity was then determined by adding 120µl/well of Apo-ONE® Homogeneous Caspase-3/7 Reagent and incubating for 0.5 hour before recording fluorescence ($485_{Ex}/527_{Em}$).

Stopping the Reaction: The fluorescence generated in the CellTiter-Blue® Assay can be stopped and stabilized by adding SDS. We recommend adding 50µl of 3% SDS per 100µl of original culture volume. The plate can then be stored at ambient temperature for up to 24 hours before recording data, provided that the contents are protected from light and covered to prevent evaporation.

Additional Resources for the CellTiter-Blue® Cell Viability Assay

Technical Bulletins and Manuals

TB317 *CellTiter-Blue® Cell Viability Assay Technical Bulletin*

EP015 *Automated CellTiter-Blue® Cell Viability Assay Protocol*

Promega Publications

Selecting cell-based assays for drug discovery screening

Multiplexing homogeneous cell-based assays

Choosing the right cell-based assay for your research

Introducing the CellTiter-Blue® Cell Viability Assay

Online Tools

Cell Viability Assistant

Citations

Bruno, I.G., Jin, W. and Cote, C.J. (2004) Correction of aberrant FGFR1 alternative RNA splicing through targeting of intronic regulatory elements. *Hum. Mol. Genet.* **13**, 2409–20.

Human U251 glioblastoma cell lines treated with antisense mopholino oligonucleotides were assessed for viability and apoptosis by multiplexing the CellTiter-Blue® Cell Viability Assay and Apo-ONE® Homogeneous Caspase-3/7 Assay on single-cell cultures.

PubMed Number: 15333583

B. Tetrazolium-Based Assays

Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce the tetrazolium product, MTS, into an aqueous, soluble formazan product. At death, cells rapidly lose the ability to reduce tetrazolium products. The production of the colored formazan product, therefore, is proportional to the number of viable cells in culture.

The CellTiter 96® AQ_{ueous} products are MTS assays for determining the number of viable cells in culture. The MTS tetrazolium is similar to the widely used MTT tetrazolium, with the advantage that the formazan product of MTS reduction is soluble in cell culture medium and does not require use of a Solubilization Solution.

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay is an MTS-based assay that involves adding a single reagent directly to the assay wells at a recommended ratio of 20µl reagent to 100µl of culture medium. Cells are incubated 1–4 hours at 37°C and then absorbance is measured at 490nm. This assay chemistry has been widely accepted and is cited in hundreds of published articles.

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation assay is also an MTS-based assay. The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Reagent is prepared by combining two solutions, MTS and an electron coupling reagent, phenazine methosulfate (PMS). The reagent is then added to cells. During the assay, MTS is converted to a soluble formazan product. Samples are read after a 1- to 4-hour incubation at 490nm.

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)

Materials Required:

- CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Cat.# G3582, G3580, G3581) and protocol #TB245
- 96-well plates suitable for tissue culture
- repeating, digital or multichannel pipettors
- 96-well spectrophotometer

General Protocol

1. Thaw the CellTiter 96® AQ_{ueous} One Solution Reagent. It should take approximately 90 minutes at room temperature on the bench top, or 10 minutes in a water bath at 37°C, to completely thaw the 20ml size.
2. Pipet 20µl of CellTiter 96® AQ_{ueous} One Solution Reagent into each well of the 96-well assay plate containing the samples in 100µl of culture medium.
3. Incubate the plate for 1–4 hours at 37°C in a humidified, 5% CO_2 atmosphere.

   Note: To measure the amount of soluble formazan produced by cellular reduction of the MTS, proceed immediately to Step 4. Alternatively, to measure the absorbance later, add 25µl of 10% SDS to each well to stop the reaction. Store SDS-treated plates protected from light in a humidified chamber at room temperature for up to 18 hours. Proceed to Step 4.
4. Record the absorbance at 490nm using a 96-well spectrophotometer.

Additional Resources for the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay

Technical Bulletins and Manuals

TB245 *CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Technical Bulletin*

Promega Publications

Selecting cell-based assays for drug discovery screening

Choosing the right cell-based sssay for your research

Online Tools

Cell Viability Assistant

Citations

Gauduchon, J. *et al.* (2005) 4-Hydroxytamoxifen inhibits proliferation of multiple myeloma cells in vitro through down-regulation of c-Myc, up-regulation of p27Kip1, and modulation of Bcl-2 family members. *Clin. Cancer Res.* **11**, 2345–54.

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay was used to evaluate cell viability of six different multiple myeloma cell lines.

PubMed Number: 15788686

Berglund, P. *et al.* (2005) Cyclin E overexpression obstructs infiltrative behavior in breast cancer: A novel role reflected in the growth pattern of medullary breast cancers. *Cancer Res.* **65**, 9727–34.

Attachment assays were performed with MDA-MB-468 cell lines stably transfected with a cyclin-E GFP fusion construct. Cells were allowed to adhere to 96-well plates, washed then incubated with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay.

PubMed Number: 16266993

CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay

Materials Required:

- CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Cat.# G5440) and protocol #TB169
- 96-well plate
- 37°C incubator
- 10% SDS

General Protocol for One 96-Well Plate Containing Cells Cultured in 100µl Volume

1. Thaw the MTS Solution and the PMS Solution.
2. Remove 2.0ml of the MTS Solution using aseptic technique and transfer to a test tube.
3. Add 100µl of PMS Solution to the 2.0ml of MTS Solution immediately before use.
4. Genetly swirl the tube to completely mix the combined MTS/PMS solution.
5. Pipet 20µl of the combined MTS/PMS solution into each well of the 96-well assay plate.
6. Incubate the plate for 1–4 hours at 37°C in a humidified, 5% CO_2 chamber.
7. Record the absorbance at 490nm using a plate reader.

Additional Resources for the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay

Technical Bulletins and Manuals

TB169 *CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay Technical Bulletin*

Promega Publications

Technically speaking: Cell viability assays

Online Tools

Cell Viability Assistant

Citations

Zhang, L. *et al.* (2004) A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A. *Mol. Cell. Biol.* **24**, 2169–80.

The cell proliferation of fetal mink lung cells was measured using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay.

PubMed Number: 14966294

Tamasloukht, M. *et al.* (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Plant Physiol.* **131**, 1468–78.

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay was used to measure metabolic activity of germinating fungal spores.

PubMed Number: 12644696

CellTiter 96® Non-Radioactive Cell Proliferation Assay

The CellTiter 96® Non-Radioactive Cell Proliferation Assay (Cat.# G4000, G4100) is a colorimetric assay system that measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by viable cells. After incubation of the cells with the Dye Solution for approximately 1–4 hours, a Solubilization Solution is added to lyse the cells and solubilize the colored product. These samples can be read using an absorbance plate reader at a wavelength of 570nm. The amount of color produced is directly proportional to the number of viable cells.

Additional Resources for the CellTiter 96® Non-Radioactive Cell Proliferation Assay

Technical Bulletins and Manuals

TB112 *CellTiter 96® Non-Radioactive Cell Proliferation Assay*

Promega Publications

Technically speaking: Cell viability assays

Online Tools

Cell Viability Assistant

Other Cell Viability Assays

The MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) is a single-reagent-addition fluorescent assay that simultaneously measures the relative number of live and dead cells in cell populations. The MultiTox-Fluor Multiplex Cytotoxicity Assay gives ratiometric, inversely correlated measures of cell viability and cytotoxicity. The ratio of viable cells to dead cells is independent of cell number and therefore can be used to normalize data. Having complementary cell viability and cytotoxicity measures reduces errors associated with pipetting and cell clumping. Assays are often subject to chemical interference by test compounds, media components and can give false-positive or false-negative results. Independent cell viability and cytotoxicity assay chemistries serve as internal controls and allow identification of errors resulting from chemical interference from test compounds or media components. More information about the MultiTox-Fluor Assay can be found in Section IV "Cytotoxicity Assays" of this chapter.

IV. Cytotoxicity Assays

A. Determining the Number of Live and Dead Cells in a Cell Population: MultiTox-Fluor Multiplex Cytotoxicity Assay

Cell-based assays are important tools for contemporary biology and drug discovery because of their predictive potential for in vivo applications. However, the same cellular complexity that allows the study of regulatory elements, signaling cascades or test compound bio-kinetic profiles also can complicate data interpretation by inherent biological variation. Therefore, researchers often need to normalize assay responses to cell viability after experimental manipulation.

Although assays for determining cell viability and cytotoxicity that are based on ATP, reduction potential and LDH release are useful and cost-effective methods, they have limits in the types of multiplexed assays that can be performed along with them. The MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) is a homogeneous, single-reagent-addition format (Figure 4.13) that allows the measurement of the relative number of live and dead cells in a cell population. This assay gives ratiometric, inversely proportional values of viability and cytotoxicity (Figure 4.15) that are useful for normalizing data to cell number. Also, this reagent is compatible with additional fluorescent and luminescent chemistries.

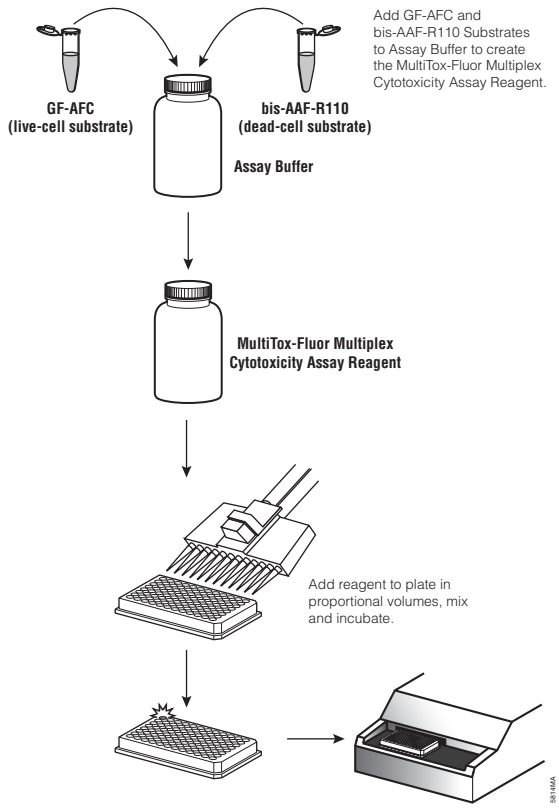

Figure 4.13. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The assay uses a homogeneous, single-reagent-addition format to determine live- and dead-cell numbers in a cell population.

The MultiTox-Fluor Multiplex Cytotoxicity Assay simultaneously measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycyl-phenylalanyl-amino-fluorocoumarin; GF-AFC). The substrate enters intact cells were it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells (Figure 4.14). This live-cell protease becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic, cell-impermeant peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity (Figure 4.14). Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

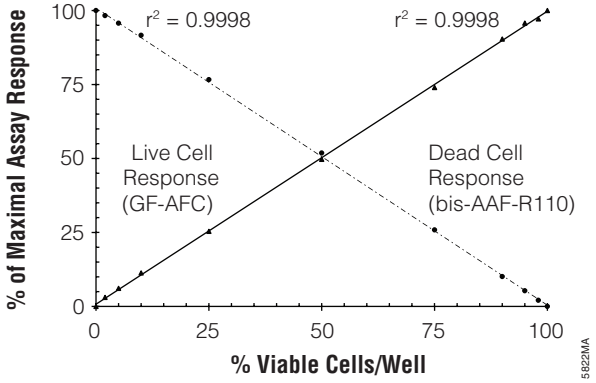

Figure 4.15. Viability and cytotoxicity measurements are inversely correlated and ratiometric. When viability is high, the live-cell signal is highest, and the dead-cell signal is lowest. When viability is low, the live-cell signal is lowest, and the dead-cell signal is highest.

MultiTox-Fluor Multiplex Cytotoxicity Assay Materials Required:

- MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) and protocol #TB348
- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reagent reservoirs
- fluorescence plate reader with filter sets: $400nm_{Ex}/505nm_{Em}$ and $485nm_{Ex}/520nm_{Em}$
- orbital plate shaker
- positive control cytotoxic reagent or lytic detergent

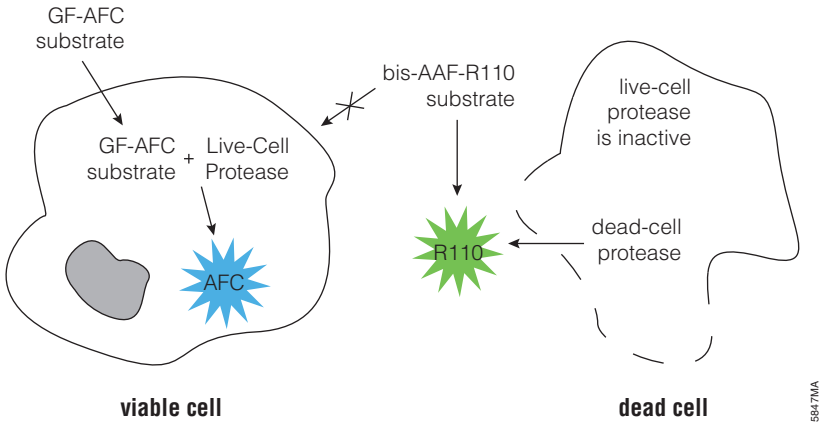

Figure 4.14. Biology of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The GF-AFC Substrate can enter live cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 Substrate cannot enter live cells, but instead can be cleaved by the dead-cell protease activity to release R110.

Example Cytotoxicity Assay Protocol

1. If you have not performed this assay on your cell line previously, we recommend determining assay sensitivity using your cells. Protocols to determine assay sensitivity are available in the *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin* #TB348.
2. Set up 96-well or 384-well assay plates containing cells in culture medium at the desired density.
3. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100µl in each well (25µl for 384-well plates).
4. Culture cells for the desired test exposure period.
5. Add MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
6. Measure the resulting fluorescence: live cells, 400nm$_{Ex}$/505nm$_{Em}$, and dead cells, 485nm$_{Ex}$/520nm$_{Em}$.

General Considerations for the MultiTox-Fluor Multiplex Cytotoxicity Assay

Background Fluorescence and Inherent Serum Activity: Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The quantity of this protease activity may vary among different lots of serum. To correct for variability, background fluorescence should be determined using samples containing medium plus serum without cells.

Temperature: The generation of fluorescent product is proportional to the protease activity of the markers associated with cell viability and cytotoxicity. The activity of these proteases is influenced by temperature. For best results, we recommend incubating at a constant controlled temperature to ensure uniformity across the plate.

Assay Controls: In addition to a no-cell control to establish background fluorescence, we recommend including an untreated cells (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by the addition of vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and the equivalent amount of solvent added with the test reagent. The maximum cytotoxicity control can be determined using a compound that causes cytotoxicity or a lytic reagent added to compromise viability (non-ionic or Zwitterionic detergents).

Cytotoxicity Marker Half-Life: The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours. In situations where cytotoxicity occurs very rapidly (necrosis) and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. The addition of a lytic detergent may be useful to determine the total cytotoxicity marker activity remaining (from remaining live cells) in these extended incubations.

Light Sensitivity: The MultiTox-Fluor Multiplex Cytotoxicity Assay uses two fluorogenic peptide substrates. Although the substrates demonstrate good general photostability, the liberated fluors (after contact with protease) can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

Cell Culture Medium: The GF-AFC and bis-AAF-R110 Substrates are introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of media formulations with either very high serum content or phenol red indicator, no substantial performance differences will be observed among media.

Additional Resources for the MultiTox-Fluor Multiplex Cytotoxicity Assay

Technical Bulletins and Manuals

TB348 *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin*

Promega Publications

Multiplexed viability, cytotoxicity and apoptosis assays for cell-based screening

MultiTox-Fluor Multiplex Cytotoxicity Assay technology

Online Tools

Cell Viability Assistant

B. Measuring the Relative Number of Dead Cells in a Population: CytoTox-Fluor™ Cytotoxicity Assay

The CytoTox-Fluor™ Cytotoxicity Assay is a single-reagent-addition, homogeneous fluorescent assay that measures the relative number of dead cells in cell populations (Figure 4.16). The CytoTox-Fluor™ Assay measures a distinct protease activity associated with cytotoxicity. The assay uses a fluorogenic peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) to measure "dead-cell protease" activity, which has been released from cells that have lost membrane integrity. The bis-AAF-R110 Substrate cannot cross the intact membrane of live cells and therefore gives no signal from live cells.

The CytoTox-Fluor™ Assay is designed to accommodate downstream multiplexing with most Promega luminescent assays or spectrally distinct fluorescent assay methods, such as assays measuring caspase activation, reporter expression or orthogonal measures of viability (Figure 4.17).

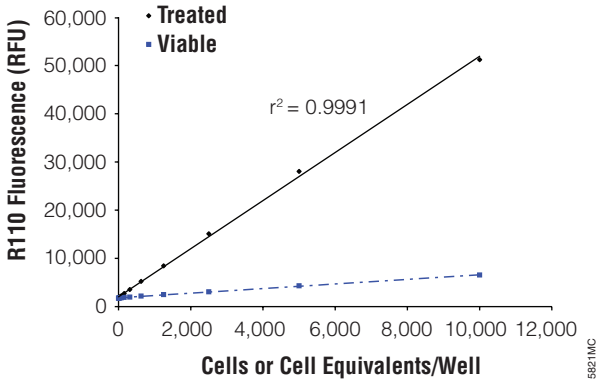

Figure 4.16. The CytoTox-Fluor™ Cytotoxicity Assay signals derived from viable cells (untreated) or lysed cells (treated) are proportional to cell number.

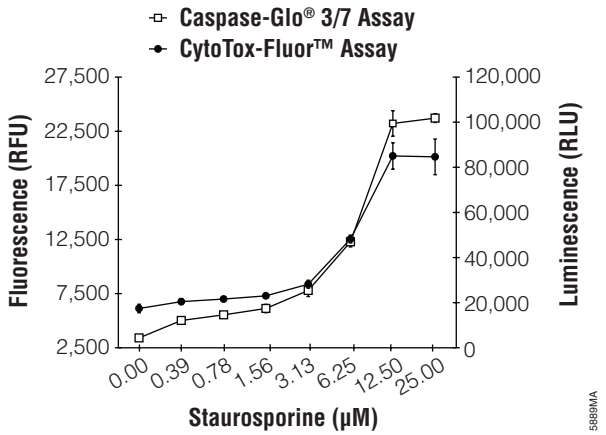

Figure 4.17. CytoTox-Fluor™ Assay multiplexed with Caspase-Glo® 3/7 Assay. The CytoTox-Fluor™ Assay Reagent is added to wells and cytotoxicity measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent is added and luminescence measured after a 30-minute incubation.

CytoTox-Fluor™ Cytotoxicity Assay
Materials Required:

- CytoTox-Fluor™ Cytotoxicity Assay (Cat.# G9206, G9207, G9208) and protocol #TB350
- 96-, 384- or 1536-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reagent reservoirs
- fluorescence plate reader with filter sets: $485nm_{Ex}/520nm_{Em}$
- orbital plate shaker
- positive control cytotoxic reagent or lytic detergent

Example Cytotoxicity Assay Protocol

1. If you have not performed this assay on your cell line previously, we recommend determining assay sensitivity using your cells. Protocols to determine assay sensitivity are available in the *CytoTox-Fluor™ Cytotoxicity Assay Technical Bulletin* #TB350.
2. Set up 96-well or 384-well assay plates containing cells in culture medium at desired density.
3. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100µl in each well (25µl for 384-well plates).
4. Culture cells for the desired test exposure period.
5. Add CytoTox-Fluor™ Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
6. Measure the resulting fluorescence: $485nm_{Ex}/520nm_{Em}$.

Example Multiplex Protocol (with luminescent caspase assay)

1. Set up 96-well assay plates contianing cells in culture medium at desired density.

2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100µl in each well (25µl for 384-well plates).
3. Culture cells for the desired test exposure period.
4. Add CytoTox-Fluor™ Cytotoxicity Assay Reagent in an equal volume to all wells, mix briefly on an orbital shaker, then incubate for 30 minutes at 37°C.
5. Measure the resulting fluorescence: $485nm_{Ex}/520nm_{Em}$.
6. Add an equal volume of Caspase-Glo® 3/7 Reagent to the wells, incubate for 30 minutes and measure luminescence.

General Considerations for the CytoTox-Fluor™ Cytotoxicity Assay

Background Fluorescence and Inherent Serum Activity: Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The quantity of this protease activity may vary among different lots of serum. To correct for variability, background fluorescence should be determined using samples containing medium plus serum without cells.

Temperature: The generation of fluorescent product is proportional to the protease activity of the marker associated with cytotoxicity. The activity of this protease is influenced by temperature. For best results, we recommend incubating at a constant controlled temperature to ensure uniformity across the plate.

Assay Controls: In addition to a no-cell control to establish background fluorescence, we recommend including an untreated cells (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by the addition of vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and the equivalent amount of solvent added with the test compound. The maximum cytotoxicity control can be determined using a compound that causes cytotoxicity or a lytic compound added to compromise viability (non-ionic or Zwitterionic detergents).

Cytotoxicity Marker Half-Life: The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours. In situations where cytotoxicity occurs very rapidly (necrosis) and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. The addition of a lytic detergent may be useful to determine the total cytotoxicity marker activity remaining (from any live cells) in these extended incubations.

Light Sensitivity: Although the bis-AAF-R110 Substrate demonstrates good general photostability, the liberated fluors (after contact with protease) can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

Cell Culture Medium: The bis-AAF-R110 Substrate is introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of media formulations with either very high serum content or phenol red indicator, no substantial performance differences will be observed among media.

Additional Resources for the CytoTox-Fluor™ Cytotoxicity Assay

Technical Bulletins and Manuals

TB350 *CytoTox-Fluor™ Cytotoxicity Assay Technical Bulletin*

Online Tools

Cell Viability Assistant

C. Cytotoxicity Assays Measuring LDH Release

Cells that have lost membrane integrity release lactate dehydrogenase (LDH) into the surrounding medium. The CytoTox-ONE™ Homogeneous Membrane Integrity Assay is a fluorescent method that uses coupled enzymatic reactions to measure the release of LDH from damaged cells as an indicator of cytotoxicity. The assay is designed to estimate the number of nonviable cells present in a mixed population of living and dead cells. Alternatively, if a cell lysis reagent is used, the same assay chemistry can be used to determine the total number of cells in a population.

LDH catalyzes the conversion of lactate to pyruvate with the concomitant production of NADH. The CytoTox-ONE™ Reagent contains excess substrates (lactate and NAD+) to drive the LDH reaction and produce NADH. This NADH, in the presence of diaphorase and resazurin, is used to drive the diaphorase-catalyzed production of the fluorescent resorufin product. Because reaction conditions proceed at physiological pH and salt conditions, the CytoTox-ONE™ Reagent does not damage living cells, and the assay can be performed directly in cell culture using a homogeneous method. The CytoTox-ONE™ Assay is fast, typically requiring only a 10-minute incubation period. Under these assay conditions, there is no significant reduction of resazurin by the population of viable cells.

The CytoTox-ONE™ Assay is compatible with 96- and 384-well formats. The detection sensitivity is a few hundred cells but can be limited by the LDH activity present in serum used to supplement culture medium. When automated on the Biomek® 2000 workstation, the CytoTox-ONE™ Assay gives excellent Z′-factor values (Figure 4.14). Because the CytoTox-ONE™ Assay is relatively nondestructive, it can be multiplexed with other assays to allow researchers to measure more than one parameter from the same sample. For multiplexing

protocols using the CytoTox-ONE™ Assay see *Cell Notes* Issue 10 or Chapter 3 "Apoptosis" of this Protocols and Applications Guide.

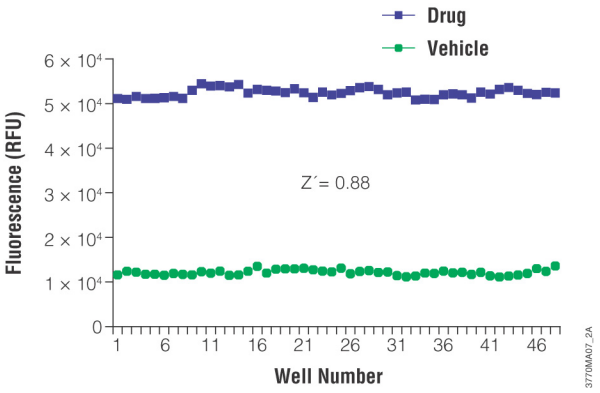

Figure 4.18. Representation of Z′-factor equal to 0.88 using the CytoTox-ONE™ Assay for one of the plates processed by the Biomek® 2000 Workstation. HepG2 cells were plated in 96-well tissue culture, treated white plates with clear bottoms at a density of 40,000 cells/well. Cells were allowed to grow to confluence and were treated with 3.125µM staurosporine on one half of the plate or DMSO vehicle control on the other half. A two-plate protocol was written for the Biomek® 2000 workstation, and plates were read on a fluorescent plate reader.

CytoTox-ONE™ Homogeneous Membrane Integrity Assay

Materials Required:

- CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cat.# G7890, G7891, G7892) and protocol #TB306
- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reservoirs to hold CytoTox-ONE™ Reagent and Stop Solution
- fluorescence plate reader with excitation 530–570nm and emission 580–620nm
- plate shaker

Example Cytotoxicity Assay Protocol

1. Set up 96-well assay plates containing cells in culture medium.
2. Add test compounds and vehicle controls to appropriate wells such that the final volume is 100µl in each well (25µl for a 384-well plate).
3. Culture cells for desired test exposure period.
4. Remove assay plates from the 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).
5. **Optional:** If the Lysis Solution is used to generate a Maximum LDH Release Control, add 2µl of Lysis Solution (per 100µl original volume) to the positive control wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.
6. Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well, and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).
7. Incubate at 22°C for 10 minutes.
8. Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl of Stop Solution. This step is optional but recommended for consistency.
9. Shake plate for 10 seconds and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm.

Calculation of Results

1. Subtract the average fluorescence values of the Culture Medium Background from all fluorescence values of experimental wells.
2. Use the average fluorescence values from Experimental, Maximum LDH Release, and Culture Medium Background to calculate the percent cytotoxicity for a given experimental treatment.

   Percent cytotoxicity = 100 × (Experimental – Culture Medium Background) / (Maximum LDH Release – Culture Medium Background)

Example Total Cell Number Assay Protocol

The CytoTox-ONE™ Assay can be used to estimate the total number of cells in assay wells at the end of a proliferation assay. The procedure involves lysing all the cells to release 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.

1. Set up 96-well assay plates containing cells in culture medium.
2. Add test compounds and vehicle controls to appropriate wells so the final volume is 100µl in each well (25µl per well for 384-well plates).
3. Culture cells for desired test exposure period.
4. Add 2µl of Lysis Solution (per 100µl of original volume) to all wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.
5. Remove assay plates from the 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).
6. Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well, and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium

containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).

7. Incubate at 22°C for 10 minutes.

8. Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well in the 96-well format. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl Stop Solution. This step is optional but recommended for consistency.

9. Shake plate for 10 seconds and record fluorescence at $560_{Ex}/590_{Em}$.

General Considerations for Performing the CytoTox-ONE™ Assay

Background Fluorescence/Serum LDH: Animal serum used to supplement tissue culture medium may contain significant amounts of LDH that can lead to background fluorescence. The quantity of LDH in animal sera will vary depending on several factors, including the species and the health or treatment of the animal prior to collecting serum. Background fluorescence can be corrected by including a control to measure the fluorescence from serum-supplemented culture medium in the absence of cells. Using reduced serum concentrations or serum-free medium can reduce or eliminate background fluorescence resulting from LDH in serum and improve assay sensitivity.

Temperature: The generation of fluorescent product in the CytoTox-ONE™ Assay is proportional to the quantity of LDH. The enzymatic activity of LDH is influenced by temperature. We recommend equilibrating the temperature of the assay plate and the CytoTox-ONE™ Reagent to 22°C (20–30 minutes) before adding the CytoTox-ONE™ Reagent to initiate the reaction. The recommended incubation period for the CytoTox-ONE™ Reagent is 10 minutes when reagents and samples are at 22°C. At longer incubation times or higher temperatures, assay linearity may decrease due to substrate depletion. In some situations, the time required for manual or robotic addition of CytoTox-ONE™ Reagent to the assay plate may be a significant portion of the 10-minute incubation period. To minimize the difference in incubation interval among wells within a plate, we recommend adding Stop Solution using the same sequence used for adding the CytoTox-ONE™ Reagent.

Assay Controls: In a standard cytotoxicity assay, a 100% cell lysis control may be performed to determine the maximum amount of LDH present. Individual laboratories may prefer to use a positive control that is known to be toxic for their specific cell type, culture conditions, and assay model system. For convenience, we include the Lysis Solution, which is a 9% (weight/volume) solution of Triton® X-100 in water. Use of Lysis Solution at the recommended dilution will result in almost immediate lysis of most cell types and subsequent release of cytoplasmic LDH into the surrounding culture medium. Use of Lysis Solution at the recommended dilution is compatible with the CytoTox-ONE™ Assay chemistry.

Considerations for the **Maximum LDH Release Control experimental design** will influence the values for the Maximum LDH Release Control. The mechanism of cytotoxicity, and thus the kinetics of release of LDH, may vary widely for different experimental compounds being tested. The method by which the Maximum LDH Release Control is prepared as well as the timing of the addition of Lysis Solution (i.e., beginning, middle or end of experimental/drug treatment period) may both affect the value obtained for 100% LDH release. For example, if the indicator cells are growing throughout the duration of exposure to test compounds, untreated control wells may have more cells and thus may have more LDH present at the end of the exposure period. Adding Lysis Solution after cultured cells are exposed to test compounds may give a different Maximum LDH Release Control value than adding Lysis Solution before the exposure period. The half-life of LDH that has been released from cells into the surrounding medium is approximately 9 hours. If Lysis Solution is added at the beginning of an experimental exposure period, the quantity of active LDH remaining in the culture medium at the end of the experiment may underestimate the quantity of LDH present in untreated cells. The recommended dilution of Lysis Solution is compatible with the enzymatic reactions and fluorescence of the assay. Using higher concentrations of Lysis Solution may increase the rate of enzymatic reactions and inflate maximum cell lysis values.

Light Sensitivity of Resazurin: The resazurin dye in the CytoTox-ONE™ Reagent and the resorufin product formed during the assay are light-sensitive. Prolonged exposure of the CytoTox-ONE™ Assay Buffer or reconstituted CytoTox-ONE™ Reagent to light will result in increased background fluorescence in the assay and decreased sensitivity.

Use of Stop Solution to Stop Development of Fluorescent Signal: The Stop Solution provided is designed to rapidly stop the continued generation of fluorescent product and allow the plate to be read at a later time. There may be situations where the researcher will want to take multiple kinetic readings of the same plate and not stop the assay. After adding the Stop Solution, provided that there is some serum (5–10%) present in the samples, the resulting fluorescence is generally stable for up to two days if the assay plate has been protected from light exposure and the wells have been sealed with a plate sealer to prevent evaporation. If no serum is present, the resulting fluorescence is stable for 1–2 hours.

Cell Culture Media: Pyruvate-supplemented medium is recommended for some cell lines. Common examples of culture media that contain pyruvate include: Ham's F12, Iscove's and some formulations of DMEM. Culture media containing pyruvate may cause a reduction in the fluorescent signal due to product inhibition of the LDH reaction catalyzing conversion of lactate to pyruvate. For most situations, the recommended assay conditions of 10 minutes at 22°C will provide adequate signal. However,

assay conditions can be empirically optimized. To increase the fluorescent signal, we recommend omitting pyruvate during the assay period, if the cell line does not require it. Alternatively, conditions known to increase the fluorescent signal include increasing the time of incubation with the CytoTox-ONE™ Reagent prior to adding Stop Solution or incubating the assay at temperatures above the recommended 22°C (up to 37°C). In all cases, all samples within a single assay should be measured using the same conditions.

Use of Resazurin as an Indicator in both Cytotoxicity and Cell Viability Assays: Resazurin reduction is a common reporter for both cytotoxicity and cell viability assays. Using the reaction conditions recommended for the CytoTox-ONE™ Assay (i.e., reduced temperature and short incubation time), only a negligible amount of resazurin is reduced by the viable cell population. In the CytoTox-ONE™ Assay, the rate of the LDH reaction is increased by providing excess substrates (pyruvate, NAD+, and diaphorase) so that the reaction proceeds relatively quickly (10 minutes at ambient temperature). By contrast, the CellTiter-Blue® Cell Viability Assay requires longer incubation times (1–4 hours) and a higher incubation temperature (37°C). Additionally, the concentration of resazurin is different between the two assays.

Additional Resources for the CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin

Technical Bulletins and Manuals

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

EP016 *Automated CytoTox-ONE™ Homogeneous Membrane Integrity Assay Protocol*

Promega Publications

Automating Promega cell-based assays in multiwell formats

Frequently asked questions: CytoTox-ONE™ Homogeneous Membrane Integrity Assay

Online Tools

Cell Viability Assistant

Citations

Chen, J. *et al.* (2003) Effect of bromodichloromethane on chorionic gondadotrophin secretion by human placental trophoblast cultures. *Toxicol. Sci.* **76**, 75–82.

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay was used to assess LDH release in trophoblasts exposed to BDCM for 25 hours.

PubMed Number: 12970577

CytoTox 96® Non-Radioactive Cytotoxicity Assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay is a colorimetric method for measuring lactate dehydrogenase (LDH), a stable cytosolic enzyme released upon cell lysis, in much the same way as [^{51}Cr] is released in radioactive assays. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader. The assay can be used to measure membrane integrity for cell-mediated cytotoxicity assays in which a target cell is lysed by an effector cell, or to measure lysis of target cells by bacteria, viruses, proteins, chemicals, etc. This assay can be used to determine general cytotoxicity or total cell number.

Two factors in tissue culture medium can contribute to background in the CytoTox 96® Assay: phenol red and LDH from animal sera. The absorbance value of a culture medium control is used to normalize the values obtained from other samples. Background absorbance from phenol red also can be eliminated by using a phenol red-free medium. The quantity of LDH in animal sera will vary depending on several parameters, including the species and the health or treatment of the animal prior to collecting serum. Human AB serum is relatively low in LDH activity, while calf serum is relatively high. The concentration of serum can be decreased to reduce the amount of LDH contribution to background absorbance. In general decreasing the serum concentration to 5% will significantly reduce background without affecting cell viability. Certain detergents (e.g., SDS and Cetrimide) can inhibit LDH activity. The Lysis Solution included with the CytoTox 96® Assay does not affect LDH activity and does not interfere with the assay. Technical Bulletin #TB163 provides a detailed protocol for performing this assay.

Additional Resources for the CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin

Technical Bulletins and Manuals

TB163 *CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin*

Promega Publications

In vitro toxicology and cellular fate determination using Promega's cell-based assays

Online Tools

Cell Viability Assistant

Citations

Hernandez, J.M. *et al.* (2003) Novel kidney cancer immunotherapy based on the granulocyte-macrophage colony-stimulating factor and carbonic anhydrase IX fusion gene. *Clin. Cancer Res.* **9**, 1906–16.

The CytoTox 96® Non-Radioactive Cytotoxicity Assay was used to determine specific cytotoxicity of human dendritic cells that were transduced with recombinant adenoviruses containing the gene encoding a fusion protein of granulocyte-macrophage colony stimulating factor and carbonic anhydrase IX.

PubMed Number: 12738749

V. Assays to Detect Apoptosis

A variety of methods are available for detecting apoptosis to determine the mechanism of cell death. The Caspase-Glo® Assays are highly sensitive, luminescent assays with a simple "add, mix, measure" protocol that can be used to detect caspase-8 (Cat.# G8200), caspase-9 (Cat.# G8210) and caspase-3/7 (Cat.# G8090) activities. If you prefer a fluorescent assay, the Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7792) is useful and, like the Caspase-Glo® Assays, can be multiplexed with other assays. A later marker of apoptosis is TUNEL analysis to identify the presence of oligonucleosomal DNA fragments in cells. The DeadEnd™ Fluorometric (Cat.# G3250) and the DeadEnd™ Colorimetric (Cat.# G7360) TUNEL Assays allow users to end-label the DNA fragments to detect apoptosis. A detailed discussion of apoptosis and methods and technologies for detecting apoptosis can be found in Chapter 3 of this Protocols & Applications Guide: Apoptosis.

VI. Multiplexing Cell Viability Assays

The latest generation of Promega cell-based assays includes luminescent and fluorescent chemistries to measure markers of cell viability, cytotoxicity and apoptosis, as well as to perform reporter analysis. Using these tools researchers can investigate how cells respond to growth factors, cytokines, hormones, mitogens, radiation, effectors, compound libraries and other signaling molecules. However, researchers often need more than one type of data from a sample, so the ability to multiplex, or analyze more than one parameter from a single sample, is desirable. Chapter three of this *Protocols & Applications Guide* presents basic protocols for multiplexing experiments using Promega homogeneous apoptosis assays. Here we present protocols for multiplexing cell viability with cytoxicity assays or reporter assays. For protocols describing multiplex experiments using cell viability and apoptosis assays, please see Chapter 3 of this guide.

The protocols provided are guidelines for multiplexing cell-based assays and are intended as starting points. As with any homogeneous assay, multiplexing assays will require researchers to optimize their assays for specific experimental systems. We strongly recommend running appropriate controls, including performing each assay individually on the samples. Additional background, optimization and control information for each assay is provided in its accompanying technical literature.

The CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7571; Technical Bulletin #TB288) is a homogeneous assay that measures ATP. This viability assay can be multiplexed with a live-cell luciferase reporter assay using the EnduRen™ Live Cell Substrate (Cat.# E6482; Technical Manual #TM244) or with the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cat.# G7891; Technical Bulletin #TB306), which assesses cytotoxicity by measuring LDH release. Figure 4.14 illustrates data obtained from a multiplexing experiment using a *Renilla* reporter assay using the EnduRen™ Live-Cell Substrate and the CellTiter-Glo® Assay.

The MultiTox-Fluor and CytoTox-Fluor™ assays can be multiplexed with luminescent assays measuring caspase activities to obtain information about apoptosis while controlling for cytotoxic or proliferative events. Example protocols for multiplex experiments using the MultiTox-Fluor or CytoTox-Fluor™ Assays with luminescent caspase assays are provided below. Additionally, the CytoTox-ONE™ Assay, which measures LDH release can be multiplexed with the Apo-ONE® Homogeneous Caspase-3/7 Assay to give information on cytotoxicity and mechanism of cell death.

A. Normalizing Reporter Gene Signal with Cell Viability

1. Culture and treat cells with the drug of interest in 90µl of medium in a 96-well plate.
2. Dilute the EnduRen™ Live Cell Substrate (Cat.# E6482) as directed in Technical Manual #TM244. Add 10µl/well of EnduRen™ Substrate (60µM) and incubate for an additional 2 hours at 37°C, 5% CO_2. You may add the EnduRen™ Substrate before or after experimental treatment, depending on cell tolerance.
3. Record luminescence to indicate reporter activity.
4. Add an equal volume of CellTiter-Glo® Reagent (100µl/well), mix for 2 minutes on an orbital shaker to induce cell lysis, and incubate an additional 10 minutes at room temperature to stabilize luminescent signal.
5. Record luminescence as described in Technical Bulletin #TB288 to indicate cell viability.

   Note: We suggest these controls: 1) Drug-treated cells with the CellTiter-Glo® Reagent alone, 2) Drug-treated cells with the EnduRen™ Substrate alone.

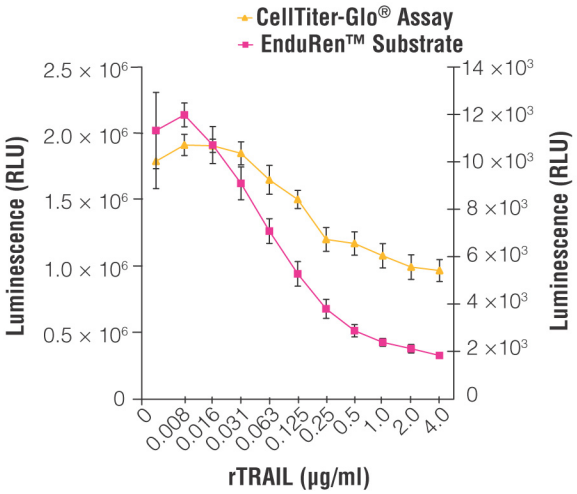

Figure 4.19. Multiplex of a *Renilla* reporter assay with a luminescent cell viability assay. HeLa cells stably expressing humanized *Renilla* luciferase were plated at 10,000 cells/well in a 96-well plate. EnduRen™ Live Cell Substrate (Cat.# E6482) was added to all of the wells at a 1:1,000 dilution in DMEM + 10% FBS. The TRAIL protein (CalBiochem Cat.# 616375) was added starting at 4µg/ml with subsequent twofold serial dilutions. Cells were incubated for 16 hours and assayed. *Renilla* expression was measured using a GloMax™ 96 Microplate Luminometer (Cat.# E6521) immediately after incubation. CellTiter-Glo® Reagent was then added at a volume of 1:1 to each well and luminescence read on the GloMax™ 96 Microplate Luminometer. RLU=Relative Light Units.

B. Determining Cytotoxicity and Cell Viability

1. Culture and treat cells with drug of interest in 75µl of medium in a 96-well plate (black or white).
2. Reconstitute CytoTox-ONE™ Substrate at 1X concentration, and add 50µl/well.
3. Shake gently and incubate for 10 minutes at room temperature. Record fluorescence ($560_{Ex}/590_{Em}$) as described in Technical Bulletin #TB306.
4. Reconstitute the CellTiter-Glo® Substrate. Add 124µl of CellTiter-Glo™ Substrate plus 1µl 20mM DTT to each well such that the final concentration of DTT in the well is 0.8mM.
5. Shake gently and incubate for 1 hour at room temperature. Record luminescence as described in Technical Bulletin #TB288.

   Note: Ensure that all of the wells change to an even pink color after incubating with CellTiter-Glo® Reagent. If all of the wells contain the same pink color when luminescence is recorded, the light is quenched evenly throughout the sample, regardless of the initial CytoTox-ONE™ Substrate activity.

C. Multiplexing CytoTox-Fluor™ Cytotoxicity Assay with a Luminescent Caspase Assay

1. Set up 96-well assay plates containing cells in culture medium at desired density.
2. Add test compounds and vehicle controls to appropriate wells so the final volume in the well is 100µl in each well (25µl for a 384-well plate).
3. Culture cells for the desired test exposure period.
4. Add 10µl CytoTox-Fluor™ Cytotoxicity Assay Reagent (prepared as 10µl substrate in 1ml Assay Buffer) to all wells, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C. **Note**: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours.
5. Measure resulting fluorescence using fluorometer ($485nm_{Ex}/520nm_{Em}$). **Note:** Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.
6. Add an equal volume of Caspase-Glo® 3/7 Reagent to wells (100–110µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

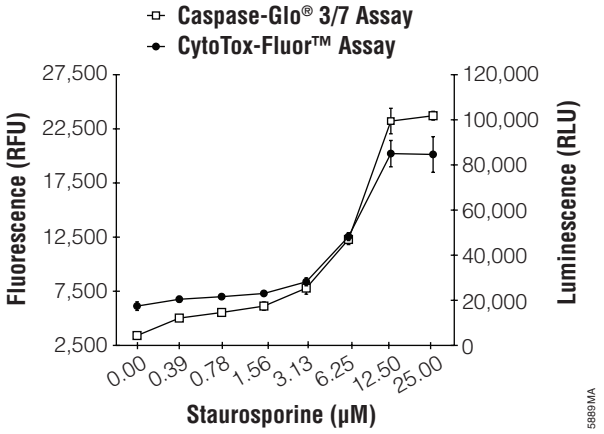

Figure 4.20. CytoTox-Fluor™ Assay multiplexed with Caspase-Glo® 3/7 Assay. The CytoTox-Fluor™ Assay Reagent is added to wells and cytotoxicity measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent is added and luminescence measured after a 30-minute incubation.

D. Multiplexing the MultiTox-Fluor Multiplex Cytotoxicity Assay with a Luminescent Caspase Assay

1. Set up 96-well assay plates containing cells in medium at the desired density.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume in the well is 100µl (25µl for a 384-well plate).
3. Culture cells for the desired test exposure period.

4. Add 10µl of MultiTox-Fluor Reagent (prepared as 10µl Substrate in 1ml Assay Buffer) to all wells, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C. **Note:** Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours.

5. Measure resulting fluorescence using a fluorometer (live-cell fluorescence $400_{Ex}/505_{Em}$; dead-cell fluorescence $485_{Ex}/520_{Em}$).

6. Add an equal volume of Caspase-Glo® 3/7 Reagent to wells (100–110µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

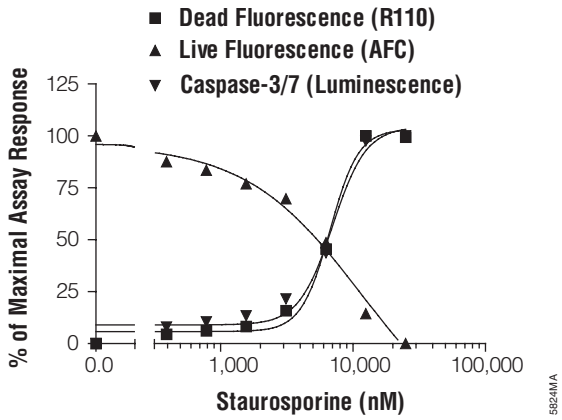

Figure 4.21. The MultiTox-Fluor Assay technology can be multiplexed with other assays . LN-18 cells were plated at a density of 10,000 cells per well in 50µl volumes of MEM + 10% fetal bovine serum and allowed to attach overnight. Staurosporine was twofold serially diluted and added to wells in 50µl volumes. The plate was incubated at 37°C in 5% CO_2 for 6 hours. MultiTox-Fluor Reagent was prepared as 10µl Substrate in 1ml Assay Buffer, and 10µl was used. The plate was mixed and incubated for 30 minutes at 37°C. Fluorescence was read on a BMG PolarStar plate reader. Caspase-Glo® 3/7 Reagent was then added in an additional 100µl volume, and luminescence measured after a 10-minute incubation. The resulting signals were normalized to a percentage of maximal response and plotted using GraphPad Prism® software.

E. Multiplexing the CytoTox-ONE™ Homogeneous Membrane Integrity Assay with the Apo-ONE® Homogeneous Caspase-3/7 Assay

1. Plate cells at the desired density (e.g., 10,000cells/well) in a white, clear-bottom 96-well plate.

2. Add treatment compound at desired concentration (e.g., tamoxifen).

3. Culture cells for the desired test exposure period.

4. To assay LDH activity transfer 50µl of the culture supernatans to a 96-well plate and add 50µl of CytoTox™ Reagent and incubate the plate for 10 minutes at 22°C. **Note:** Pyruvate in the cell-culture medium can inhibit the LDH reaction. Cells in medium supplemented with pyruvate may require a longer incubation.

5. Stop the reaction by adding 25µl of Stop solution to each well.

6. Measure fluorescence at $560_{Em}/590_{Ex}$.

7. To determine the activity of caspase-3/7, add 50µl of Apo-ONE® Reagent to the original culture plate containing cells, and incubate at room temperature for 45 minutes.

8. Measure fluorescence at $485_{Ex}/527_{Em}$.

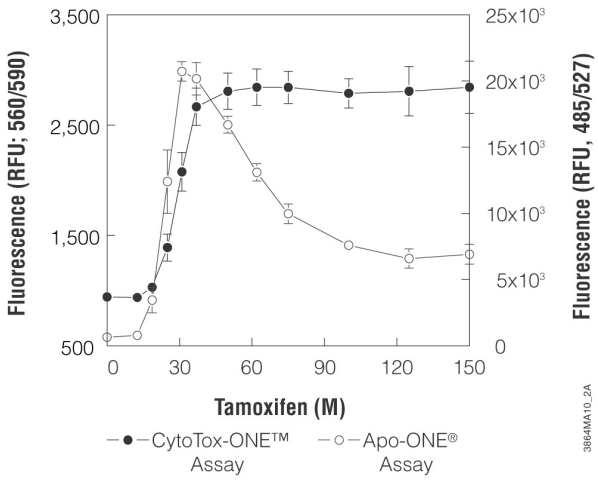

Figure 4.22. Example data showing multiplexed caspase-3/7 and LDH release assays using different plates. HepG2 cells were plated at 10,000 cells/well in a white, clear-bottom 96-well plate and cultured overnight. Various concentrations of tamoxifen were added to the wells and incubated for 4 hours at 37°C. To assay LDH activity, 50µl/well culture supernatants were transferred to a 96-well plate to which 50µl/well of CytoTox-ONE™ Reagent was added. LDH samples were incubated at ambient temperature for 30 minutes prior to stopping with 25µl/well of Stop Solution. Fluorescence 560/590nm was determined. For caspase-3/7 determination, 50µl/well of Apo-ONE® Reagent was added to the original culture plate containing cells, and incubated at ambient temperature for 45 minutes prior to determining fluorescence 485/527nm. (**Note:** The 30-minute incubation period for the assay was longer than the 10 minutes recommended in the technical bulletin because the medium for these cells was supplemented with pyruvate (which inhibits the LDH reaction) and the assay was performed at ambient temperature, which was a little colder than the 22°C recommended in the technical bulletin.

Promega Publications

Perform multiplexed cell-based assays on automated platforms

Multiplexing homogeneous cell-based assays

VII. References

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