

## SELECTING CELL-BASED ASSAYS FOR DRUG DISCOVERY SCREENING

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### Introduction

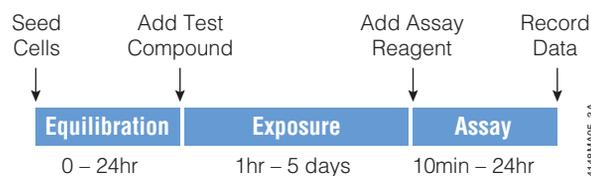
Pharmaceutical companies approach drug discovery in a variety of ways. An early part of the experimental process often involves screening a large number of compounds using defined biochemical assays in an ultrahigh-throughput format. However, the effect of a drug on an organism is complex and involves interactions at multiple levels that cannot be predicted using biochemical assays. Trying to understand this complexity has contributed to an increased use of cell-based screening assays as more biologically relevant surrogates to predict the response of the organism. In addition, at some point in the drug discovery process, predicting cellular toxicity is important. Eukaryotic cell culture is accepted as the model system of choice to get a first approximation of toxicity. Furthermore, advances in assay chemistries and signal detection technology have allowed miniaturization of cell-based assays, making it more convenient to perform dose-response experiments during primary screens.

### Promega produces a complete portfolio of homogeneous “add-mix-measure” assays...

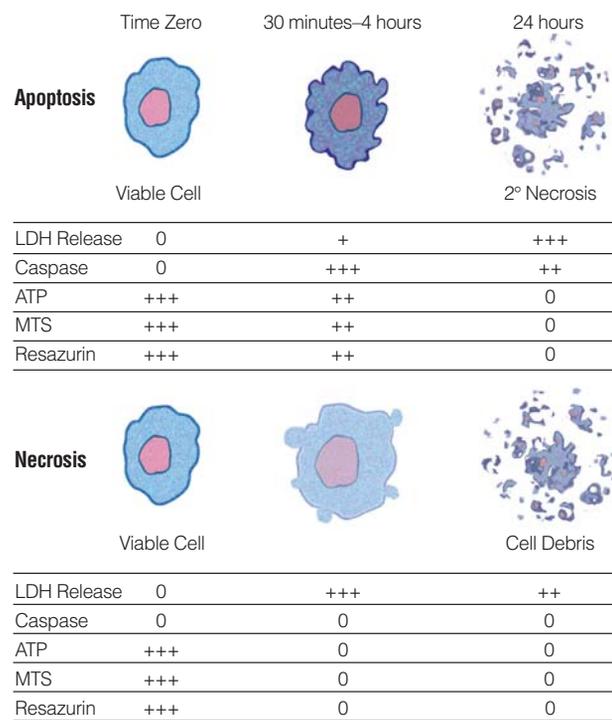
Choosing a cell-based screening assay from among the available options requires understanding the endpoint measured, the correlation with cell viability, and the limitations of the assay chemistries. Here we provide recommendations for characterizing a model assay system, describe some of the factors to consider when choosing cell-based assays for automated systems and show examples of multiplexing cell-based assays.

### 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. Choosing a biologically representative cell line and appropriate assay conditions are important for providing relevant results. 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 before the assay affects responsiveness to toxic compounds (1). Maintenance and handling of stock cultures at each step of the 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 scale up assay throughput by changing from 96- to 384- or 1536-well formats.



**Figure 1. Generalized scheme representing an in vitro cytotoxicity assay protocol.**



**Figure 2. The Promega portfolio of cell-based assays allows researchers to monitor cell viability using a variety of cellular markers. See Table 1 for more information.**

### Choosing an Endpoint to Measure

One of the first things to decide before choosing an assay format 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).

A basic understanding of the changes that occur during different mechanisms of cell death will help in deciding which endpoint to choose for a cytotoxicity assay (Figure 2). Apoptotic cells in culture eventually undergo secondary

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**Table 1. Comparison of Promega Homogeneous Cell Viability and Apoptosis Assays.**

	CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	CellTiter-Blue® Cell Viability Assay (Resazurin)	CellTiter 96® AQUEOUS One Solution Assay (MTS)	CytoTox-ONE™ Membrane Integrity Assay (LDH)	Apo-ONE® Homogeneous Caspase-3/7 Assay	Caspase-Glo® 3/7, 8 or 9 Assays
Incubation Time	10 minutes	1–4 hours	1–4 hours	10 minutes	1–18 hours	30–120 minutes
Parameter Measured	ATP	Resazurin (reducing potential)	MTS (reducing potential)	LDH Release	Caspase Activity	Caspase Activity
Detection Method	Bioluminescent	Fluorometric	Colorimetric	Fluorometric	Fluorometric	Bioluminescent
Sensitivity*					Several hundred	
96-well format	50 cells	390 cells	800 cells	800 cells	cells in a	20 cells
384-well format	15 cells	50 cells	200 cells	200 cells	population	20 cells

\*Sensitivity is dependent on cell type and other experimental conditions.

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 expressed 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.

If the information sought from your assay 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 desired, the duration of exposure to toxin, the concentration of the test compound, and the choice of the assay endpoint become critical.

## Characterizing Assay Responsiveness

Protocols used to measure cytotoxicity in vitro differ widely (Figure 1). Assay plates may be set up containing cells and equilibrated for a predetermined period before adding test compounds, or cells may be added directly to plates that already contain library compounds. The duration of exposure to the toxin may vary, 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. Assay protocols that require only a few minutes to generate a measurable signal (e.g., ATP quantitation or LDH-release assays) represent a snapshot in time and have an advantage over assays that may require one to four hours of incubation to develop a signal (e.g., MTS or resazurin). Additionally, rapid assays reduce the chance of artifacts caused by test compounds interacting with assay chemistry.

In vitro cultured cells exist as a heterogeneous population even when grown under controlled conditions. 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.

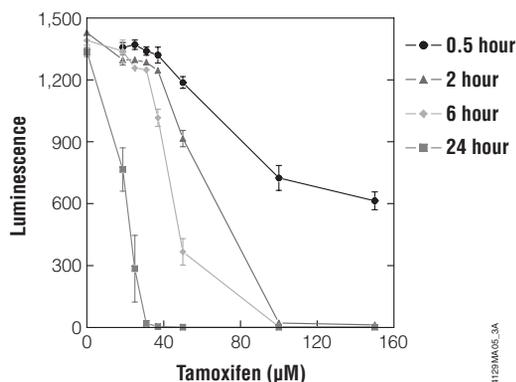
## Determining Dose and Duration of Exposure

Characterizing assay responsiveness for each in vitro model system is important, especially when trying to distinguish between mechanisms of cell death. Initial experiments should determine the appropriate assay window using an established positive control. Figures 3 and 4 show the results of two such experiments to determine the kinetics of cell death caused by different concentrations of tamoxifen treatment of HepG2 cells using two different endpoints: ATP as an indicator of viability and caspase activity as a marker for apoptosis.

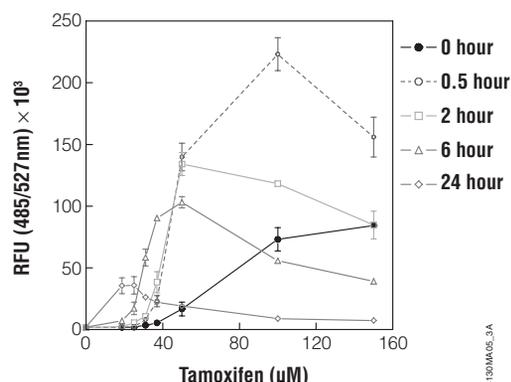
The ATP data in Figure 3 indicate that high concentrations of tamoxifen are toxic after only one half-hour exposure. The longer the duration of tamoxifen exposure, the lower the IC<sub>50</sub> value, suggesting the occurrence of a cumulative cytotoxic effect. Both the concentration of toxin and the duration of exposure contribute to the cytotoxic effect.

Some apoptosis markers are transient and may be detectable only within a limited window of time. The data from the caspase assay in Figure 4 illustrate the transient nature of caspase activity in apoptotic cells. The total amount of caspase activity measured after 24 hours of exposure to tamoxifen is only a fraction of earlier time points. There is a similar trend of shifting to lower IC<sub>50</sub> 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 24 hours of

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**Figure 3. Characterization of the toxic effects of tamoxifen on HepG2 cells using the CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay to measure ATP as an indicator of cell viability.** Legend indicates duration of tamoxifen exposure.



**Figure 4. Characterization of the toxic effects of tamoxifen on HepG2 cells using the Apo-ONE<sup>®</sup> Assay to measure caspase-3/7 activity as a marker of apoptosis.** Legend indicates duration of tamoxifen exposure.

exposure most of the population of cells are in a state of secondary necrosis.

## Homogeneous Assays for Automated Screening

Promega produces a complete portfolio of homogeneous “add-mix-measure” assays that are designed to meet a variety of screening requirements. Some of the assay systems require combining components to create the “Reagent,” and some protocols require incubation or agitation steps, but none require removal of buffer or medium from assay wells. The available homogeneous systems include assays to measure cell viability, cytotoxicity, apoptosis and reporter gene response.

## ATP Assay of Cell Viability

The amount of ATP in cells correlates with cell viability. Within minutes after loss of membrane integrity, cells lose the ability to synthesize ATP; endogenous ATPases destroy any remaining ATP, and ATP levels fall precipitously. The CellTiter-Glo<sup>®</sup> 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 CellTiter-Glo<sup>®</sup> Reagent does three things upon addition to cells. It lyses cell membranes to release ATP; it inhibits endogenous ATPases, and it provides luciferin and luciferase necessary to measure ATP using a bioluminescent reaction. The “glow-type” signal of the proprietary Ultra-Glo<sup>™</sup> Luciferase 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<sup>®</sup> Assay can detect as few as 15 cells (Table 1). Although equilibration of assay plates to room temperature is recommended before performing the assay, the assay can be completed rapidly. The luminescent signal can be detected as soon as 10 minutes after adding reagent or several hours

later for batch processing of plates. Example data are shown above in Figure 3. Among the homogeneous viability assays, the ATP assay is the fastest to perform and can detect the smallest number of cells, making it useful for 384- and 1,536-well formats.

## Tetrazolium Reduction Cell Viability Assay

The CellTiter 96<sup>®</sup> AQ<sub>UEOUS</sub> One Solution Cell Proliferation Assay is the industry standard for homogeneous colorimetric cell viability assays (2,3). Viable cells convert the MTS tetrazolium reagent into a colored formazan product during a 1- to 4-hour incubation. The amount colored formazan product is directly proportional to the number of viable cells (4).

## Resazurin Reduction Cell Viability Assay

The CellTiter-Blue<sup>®</sup> Cell Viability Assay uses an optimized reagent containing resazurin, which is reduced to fluorescent resorufin in living cells. The reagent is added directly to cells in culture, incubated, and the signal is read using a multiwell fluorometer. Because different cell types have different abilities to reduce resazurin, optimizing incubation time with the CellTiter-Blue<sup>®</sup> Reagent can improve assay sensitivity for a given model system. The detection sensitivity is intermediate between the ATP assay and the MTS reduction assay (Table 1). The simple, inexpensive procedure can be multiplexed with other assays to collect a variety of data (2,5). The assay provides good Z'-factor values in HTS situations as well (6).

## LDH-Release Cytotoxicity Assay

Cells that have lost membrane integrity release lactate dehydrogenase (LDH) into the surrounding medium. The CytoTox-ONE<sup>™</sup> 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

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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.

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, and is compatible with 96- and 384-well formats. The detection sensitivity is a few hundred cells (Table 1) 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 gave excellent Z'-factor values (7).

## Fluorescent Caspase-3/7 Assay to Detect Apoptosis

The activity of executioner caspases such as caspase-3 and -7 is an accepted, reliable indicator of apoptosis. The Apo-ONE® Homogeneous Caspase-3/7 Assay detects caspase-3/7 activity based on the cleavage of a profluorescent DEVD peptide-rhodamine 110 substrate. The Apo-ONE® Reagent is prepared by combining buffer and substrate and adding it directly to culture wells using a 1:1 ratio of reagent to medium, mixing and incubating. The reagent permeabilizes the cells to release the caspase, delivers the profluorescent substrate, and provides optimized conditions to stabilize caspase activity.

Because the fluorescent R110 product continues to accumulate in the presence of active caspase-3 and -7, extending the incubation period up to 18 hours increases the signal-to-background ratio, providing greater sensitivity. The Apo-ONE® Assay is easily scalable for HTS as long as the 1:1 ratio of reagent to medium is maintained. The detection sensitivity is in the range of several hundreds of cells (Table 1) but can be influenced by the length of incubation. An example set of data from the Apo-ONE® Assay is shown in reference 7.

## Luminescent Caspase Assays to Detect Apoptosis

The Caspase-Glo® 3/7, 8 and 9 Assays measure caspase activity based on the cleavage of a peptide-aminoluciferin substrate. Caspase cleavage of the substrate liberates free aminoluciferin, which can be used as a substrate by luciferase to generate light. The Caspase-Glo® Reagent is prepared by combining a lyophilized substrate and buffer. The reagent is added directly to cells in culture at a 1:1 ratio of reagent to medium, mixed and incubated, and luminescence is recorded. The assay has a flexible incubation time for recording the “glow-type” luminescent signal. When steady state is reached after approximately 30 minutes to one hour of incubation, the luminescent signal of this coupled enzymatic assay is directly proportional to the amount of caspase over a broad linear range (7–9). The Caspase-Glo® Assays are the most sensitive caspase assays available (Table 1). Because they are luminescent assays, fluorescent compounds will not interfere with results.

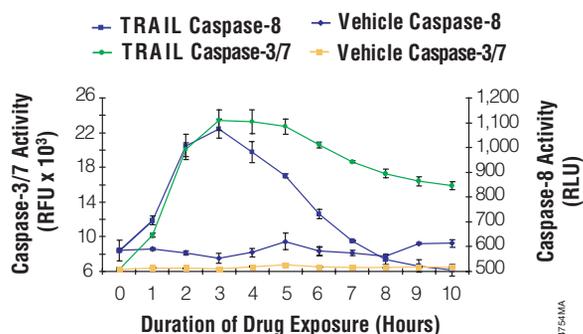


Figure 5. Multiplex measurement of two proteases (Caspase-3/7 and -8) simultaneously using the Apo-ONE® Assay and the Caspase-Glo® 8 Assay.

## Other Factors to Consider When Choosing Assays

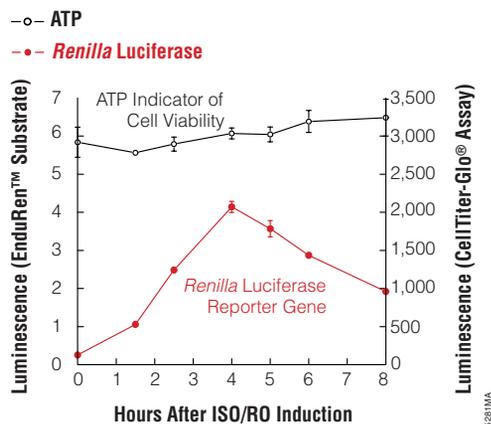
A primary concern for many researchers is **ease of use**. Homogeneous assays do not require removal of culture medium, cell washes or centrifugation steps. When choosing an assay, the time required for reagent preparation and the total length of time necessary to develop a signal from the assay chemistry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

**Sensitivity of detection** will vary with cell type if you choose to measure a metabolic marker, such as ATP levels or MTS tetrazolium reduction. The sensitivity of some assays may improve by increasing incubation time, resulting in increased signal-to-background ratios. The sensitivity depends upon the parameter measured but also on other characteristics 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. On the other hand, assay model systems that use low cell numbers in 1536-well plates may require maximum sensitivity of detection above background such as 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. In addition, the signal generated by the assay also should 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. 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.

The **reproducibility of data** is an important consideration when choosing a commercial assay. However, for most cell-based

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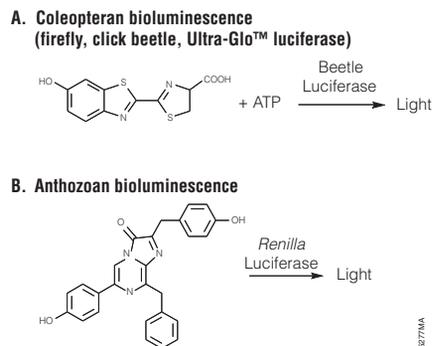
**Figure 6. Multiplexing a *Renilla* reporter assay with the CellTiter-Glo® Assay (ATP-based cell viability assay).**

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 cell lines that tend to grow in clumps rather than remain a suspension of individual cells. Extended incubation periods and edge effects in plates may also lead to decreased reproducibility among replicates and decreased or poor Z'-factor values.

### More Than One Set of Data from One Sample

The ability to gather more than one set of data from the same sample (i.e., multiplexing) can contribute to saving time and effort during screening. Multiplexing can provide internal normalization controls to confirm the results of other assay methods and eliminate the need to repeat work. Some general requirements for multiplexing include that the detection signals of the different assays are distinguishable from each other and that the assay chemistries must be compatible or separable in time and/or location.

Fluorophores that emit at different wavelengths have been used extensively to distinguish among multiple signals. Recently, there have been a growing number of examples using luminescence for multiplexing either in combination with other luminescent signals or in combination with fluorescence. (See article on page 5 of this issue for examples of Dual-Luciferase® screening assays for GPCR modulators). For example, the Chroma-Glo™ Luciferase Assay System is designed to generate luminescence from luciferases emitting two different colors (red and green) within a single sample after adding a single reagent. Use of the appropriate filter set enables the red and green luminescence produced by Chroma-Luc™ luciferases to be measured independently (10).



**Figure 7. Coleopteran and *Renilla* luciferin substrates.**

Recording two different luminescent signals (without wavelength restrictions) in sequence can be used for multiplexing if the first signal is adequately quenched to avoid interference with the second signal. For example, the Dual-Glo™ Luciferase Assay System is designed to allow high-throughput analysis of mammalian cells containing genes for firefly and *Renilla* luciferases, grown in 96- or 384-well plates. The Dual-Glo™ Luciferase Reagent can be added directly to cells in growth medium without washing or preconditioning. This reagent induces cell lysis and acts as a substrate for firefly luciferase. Addition of Dual-Glo™ Stop & Glo® Reagent quenches the luminescence from the firefly reaction by at least 10,000-fold and provides a substrate for *Renilla* luciferase in a reaction that also can be read within 2 hours (10).

### Multiplexing Luminescent and Fluorescent Assays

An example of combining luminescence and fluorescence for multiplex measurement of two proteases simultaneously in the same sample well is shown in Figure 5. An upstream signaling caspase-8 is measured using a luminescent signal, and the executioner caspase-3/7 activity is measured using a fluorescent signal. This assay combines a luminogenic substrate for caspase-8 and a pro-fluorescent substrate for caspase-3/7 in the same solution to enable a homogeneous single-reagent-addition protocol.

### Multiplexing Reporter with Cell Viability Assays

The EnduRen™ and ViviRen™ Live Cell Substrates are non-destructive assays that allow repeated *Renilla* reporter gene activity measurements over a period of time and enable multiplexing with several different types of assays (11). Measuring cell viability can be used to normalize data and distinguish the difference between specific downregulation of a reporter gene expression and a nonspecific toxic effect on the cells. Figure 6 shows an example of multiplexing a *Renilla* reporter assay and the ATP viability assay.

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## Chemistry Compatibility

The two generalized equations in Figure 7 depict some of the main components necessary to use luciferase assay chemistries. A basic understanding of these equations and the reactants necessary to drive the enzymatic reactions is useful for designing multiplex assays.

For example, the CellTiter-Glo® Reagent designed to measure small amounts of ATP contains an excess amount of both luciferin and beetle luciferase to drive the generation of luminescence. On the other hand, genetic reporter assay reagents designed to measure the presence of beetle luciferase contain an excess of both luciferin and ATP. The ATP viability assay and the beetle luciferase reporter gene assay are not compatible for multiplexing because the reagent for one assay contains an excess of exactly what the other assay is trying to measure. However, it is still possible to multiplex a luminescent reporter gene assay and ATP viability assay by using *Renilla* luciferase as the genetic reporter. As shown in the equation in Figure 7, *Renilla* luciferase does not use ATP for the catalysis of coelenterazine to generate light. Figure 6 shows an example of combining a *Renilla* luciferase reporter gene assay and an ATP cell viability assay to measure both parameters in the same sample well.

## Separating Assay Chemistries for Multiplexing

There are some situations where multiplexing of incompatible assay chemistries can be achieved by performing the assays in separate plates. Although nonhomogeneous protocols are required, the advantages of multiplexing two assays using the same sample of cells can be achieved by separating the incompatible assay chemistries into separate assay plates. This is easily accomplished when the marker to be measured is cell permeable or is released into the culture medium.

For example, if the resazurin reduction chemistry of the fluorescent LDH-release assay is not compatible with the secondary assay chemistry, a small aliquot of culture medium can be removed to a separate plate to run the LDH assay. This separation technique leaves the original sample of cells unaltered by addition of detection reagents and available for any other assay including gene reporter, image analysis, etc (2).

## Summary

Choosing the right cell-based assay and endpoint is important for generating high-quality data that answer your research questions with the greatest accuracy, speed and efficiency. The range of homogeneous luminescent and fluorescent cell-based assays available from Promega gives researchers the flexibility and tools needed to design multiplexed assays to efficiently dissect complex cellular processes. ■

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## Protocols

Are available as PDF files at:  
[www.promega.com/tbs/](http://www.promega.com/tbs/)

## Ordering Information

Product	Size	Cat.#
Caspase-Glo® 8 Assay*	100ml	G8202
Caspase-Glo® 9 Assay*	100ml	G8212
Caspase-Glo® 3/7 Assay*	100ml	G8092
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml	G7791
CellTiter-Glo® Luminescent Cell Viability Assay	10 × 10ml	G7571
CellTiter-Blue® Cell Viability Assay	20ml	G8080
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP	1,000–4,000 assays	G7892
CellTiter 96® AQueous One Solution Cell Proliferation Assay*	5,000 assays	G3581
EnduRen™ Live Cell Substrate	3.4mg	E6482
ViviRen™ Live Cell Substrate	3.7mg	E6492

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