Cell Viability Assays

Terry L Riss, PhD,[1,*] Richard A Moravec, BS,[1] Andrew L Niles, MS,[1] Sarah Duellman, PhD,[1] Hélène A Benink, PhD,[1] Tracy J Worzella, MS,[1] and Lisa Minor[2,†]

Created: May 1, 2013; Updated: July 1, 2016.

Abstract

This chapter is an introductory overview of the most commonly used assay methods to estimate the number of viable cells in multi-well plates. This chapter describes assays where data are recorded using a plate-reader; it does not cover assay methods designed for flow cytometry or high content imaging. The assay methods covered include the use of different classes of colorimetric tetrazolium reagents, resazurin reduction and protease substrates generating a fluorescent signal, the luminogenic ATP assay, and a novel real-time assay to monitor live cells for days in culture. The assays described are based on measurement of a marker activity associated with viable cell number. These assays are used for measuring the results of cell proliferation, testing for cytotoxic effects of compounds, and for multiplexing as an internal control to determine viable cell number during other cell-based assays.

Introduction

Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that

1 Promega Corporation; Email: Terry.riss@promega.com; Email: Rich.moravec@promega.com; Email: Andrew.niles@promega.com; Email: Helene.benink@promega.com; Email: Tracy.worzella@promega.com. **2** In Vitro Strategies, LLC; Email: lisakminor@yahoo.com.

Corresponding author.

* Editor
† Editor

NLM Citation: Riss TL, Moravec RA, Niles AL, et al. Cell Viability Assays. 2013 May 1 [Updated 2016 Jul 1]. In: Sittampalam GS, Coussens NP, Brimacombe K, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004-.

eventually lead to cell death. Cell-based assays also are widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a variety of assay methods that can be used to estimate the number of viable eukaryotic cells. This chapter will provide an overview of some of the major methods used in multi-well formats where data are recorded using a plate reader. The methods described include: tetrazolium reduction, resazurin reduction, protease markers, and ATP detection. Methods for flow cytometry and high content imaging may be covered in different chapters in the future.

The tetrazolium reduction, resazurin reduction, and protease activity assays measure some aspect of general metabolism or an enzymatic activity as a marker of viable cells. All of these assays require incubation of a reagent with a population of viable cells to convert a substrate to a colored or fluorescent product that can be detected with a plate reader. Under most standard culture conditions, incubation of the substrate with viable cells will result in generating a signal that is proportional to the number of viable cells present. When cells die, they rapidly lose the ability to convert the substrate to product. That difference provides the basis for many of the commonly used cell viability assays. The ATP assay is somewhat different in that the addition of assay reagent immediately ruptures the cells, thus there is no incubation period of reagent with a viable cell population.

Tetrazolium Reduction Assays

A variety of tetrazolium compounds have been used to detect viable cells. The most commonly used compounds include: MTT, MTS, XTT, and WST-1. These compounds fall into two basic categories: 1) MTT which is positively charged and readily penetrates viable eukaryotic cells and 2) those such as MTS, XTT, and WST-1 which are negatively charged and do not readily penetrate cells. The latter class (MTS, XTT, WST-1) are typically used with an intermediate electron acceptor that can transfer electrons from the cytoplasm or plasma membrane to facilitate the reduction of the tetrazolium into the colored formazan product.

MTT Tetrazolium Assay Concept

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS) (1). The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/ml, and incubated for 1 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. A reference wavelength of 630 nm is sometimes used, but not necessary for most assay conditions.

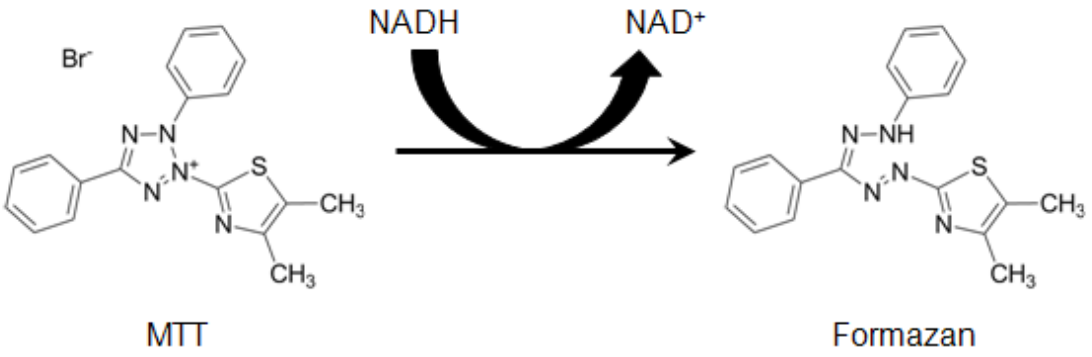

Figure 1: Structures of MTT and colored formazan product.

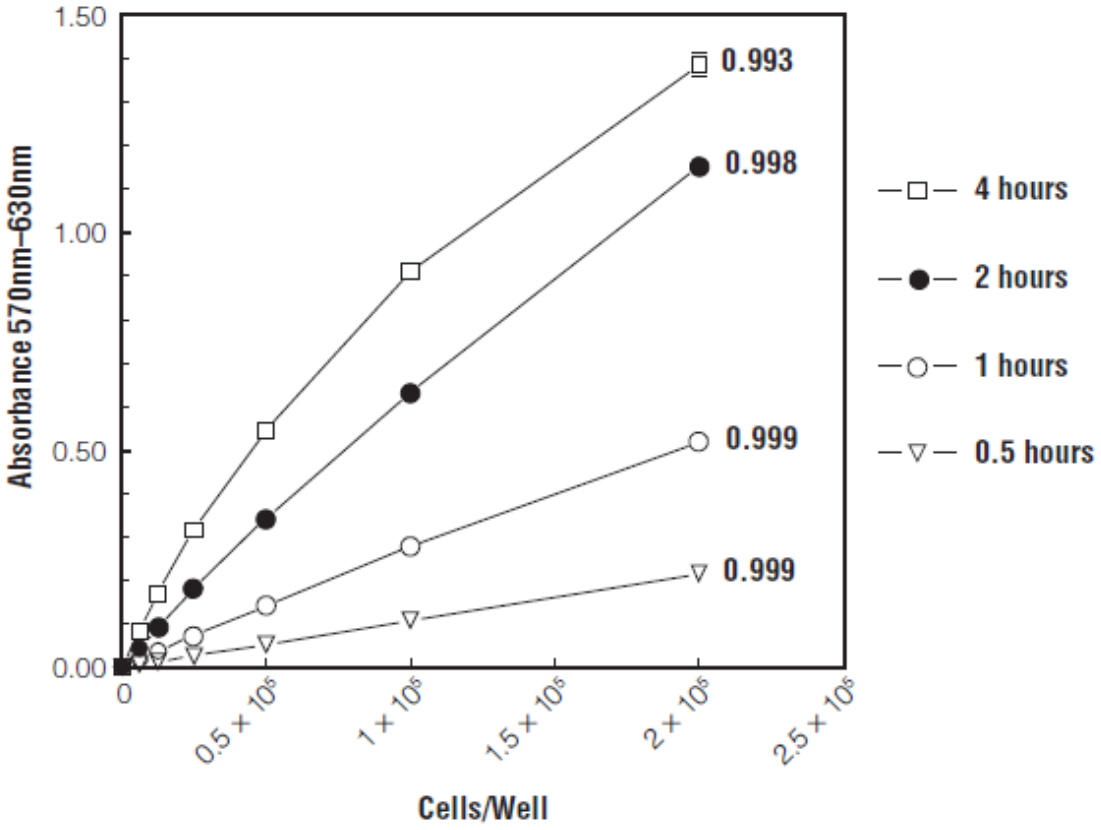

Figure 2: Direct correlation of formazan absorbance with B9 hybridoma cell number and time-dependent increase in absorbance. Note: there is little absorbance change between 2 and 4 hours. Adapted from CellTiter 96® Non-Radioactive Cell Proliferation Assay Technical Bulletin #112 (9).

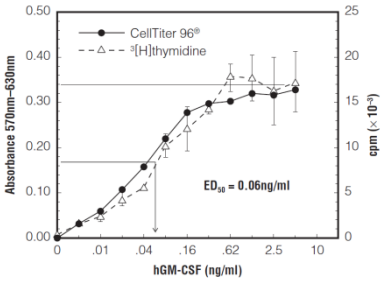

Figure 3: A comparison of using the MTT and ^{3}[H]thymidine incorporation assays of hGM-CSF-treated TF-1 cells. A blank absorbance value of 0.065 (from wells without cells but treated with MTT) was subtracted from all absorbance values. Adapted from CellTiter 96® Non-Radioactive Cell Proliferation Assay Technical Bulletin #112 (9).

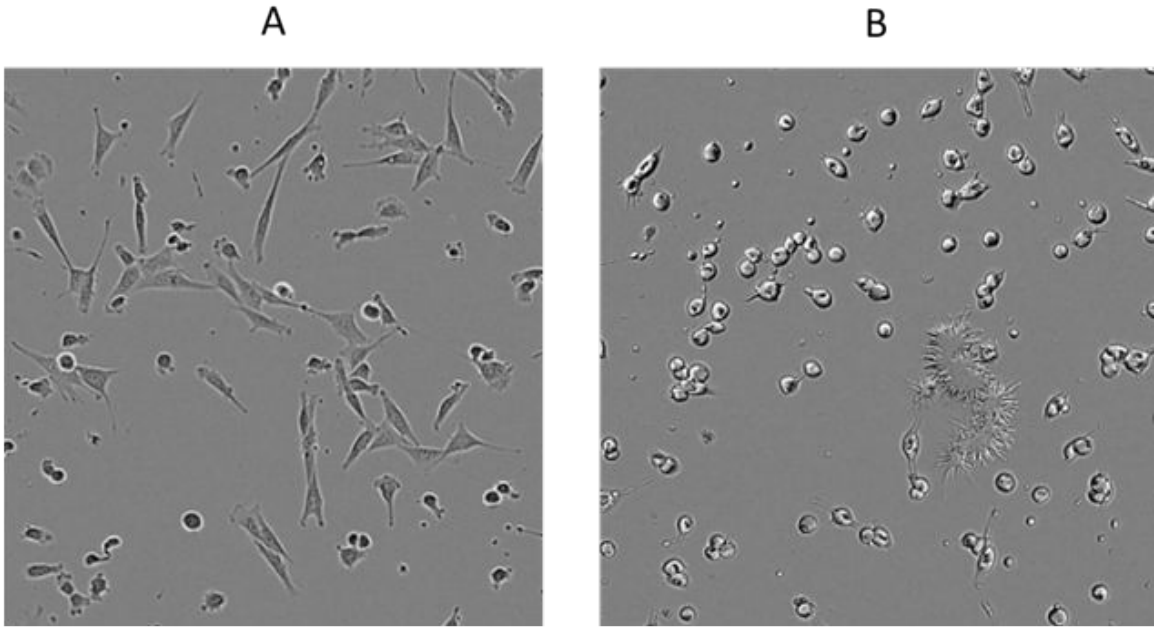

Figure 4: Change in NIH3T3 cell morphology after exposure to MTT (0.5 mg/ml). Panel A shows a field of cells photographed immediately after addition of the MTT solution. Panel B shows the same field of cells photographed after 4 hours of exposure to MTT. Panel B shows a change in cell morphology and the appearance of formazan crystals. Images were captured using the IncuCyte™ FLR from Essen Biosciences.

Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm (Figure 1). When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (2). Speculation in the early

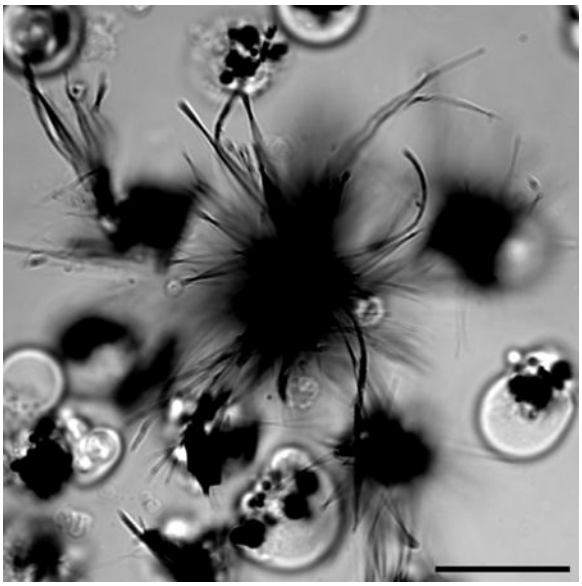

Figure 5: U937 cells incubated with MMT tetrazolium for 3 hours showing formazan crystals larger than the cells. Image was captured using an Olympus FV500 confocal microscope. Scale bar = 20 μm

literature involving specific mitochondrial enzymes has led to the assumption mentioned in numerous publications that MTT is measuring mitochondrial activity (3, 4).

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the culture medium. The formazan must be solubilized prior to recording absorbance readings. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components (5-7). Various solubilization methods include using: acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent (1, 5-7). Acidification of the solubilizing solution has the benefit of changing the color of phenol red to yellow color that may have less interference with absorbance readings. The pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is an issue (8); however, other assay technologies offer much greater sensitivity than MTT.

The amount of signal generated is dependent on several parameters including: the concentration of MTT, the length of the incubation period, the number of viable cells and their metabolic activity. All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of product that can be detected above background.

The conversion of MTT to formazan by cells in culture is time dependent (Figure 2).

Longer incubation time will result in accumulation of color and increased sensitivity up to a point; however, the incubation time is limited because of the cytotoxic nature of the

detection reagents which utilize energy (reducing equivalents such as NADH) from the cell to generate a signal. For cell populations in log phase growth, the amount of formazan product is generally proportional to the number of metabolically active viable cells as demonstrated by the linearity of response in Figure 2. Culture conditions that alter the metabolism of the cells will likely affect the rate of MTT reduction into formazan. For example, when adherent cells in culture approach confluence and growth becomes contact inhibited, metabolism may slow down and the amount MTT reduction per cell will be lower. That situation will lead to a loss of linearity between absorbance and cell number. Other adverse culture conditions such as altered pH or depletion of essential nutrients such as glucose may lead to a change in the ability of cells to reduce MTT.

The MTT assay was developed as a non-radioactive alternative to tritiated thymidine incorporation into DNA for measuring cell proliferation (1). In many experimental situations, the MTT assay can directly substitute for the tritiated thymidine incorporation assay (Figure 3).

However, it is worth noting that MTT reduction is a marker reflecting viable cell metabolism and not specifically cell proliferation. Tetrazolium reduction assays are often erroneously described as measuring cell proliferation without the use of proper controls to confirm effects on metabolism (10).

Shortly after addition of MTT, the morphology of some cell types can be observed to change dramatically suggesting altered physiology (11 and Figure 4).

Toxicity of the MTT compound is likely related to the concentration added to cells. Optimizing the concentration may result in lower toxicity. Given the cytotoxic nature of MTT, the assay method must be considered as an endpoint assay. A recent report speculated that formazan crystals contribute to harming cells by puncturing membranes during exocytosis (12). The observation of extracellular formazan crystals many times the diameter of cells that grow longer over time make it seem unlikely that exocytosis of those large structures was involved (Figure 4 and 5).

Growing crystals may suggest that marginally soluble formazan accumulates where seed crystals have begun to deposit.

Reducing compounds are known to interfere with tetrazolium reduction assays. Chemicals such as ascorbic acid, or sulfhydryl-containing compounds including reduced glutathione, coenzyme A, and dithiothreitol, can reduce tetrazolium salts non-enzymatically and lead to increased absorbance values in assay wells (13-17). Culture medium at elevated pH or extended exposure of reagents to direct light also may cause an accelerated spontaneous reduction of tetrazolium salts and result in increased background absorbance values. Suspected chemical interference of test compounds can be confirmed by measuring absorbance values from control wells without cells incubated with culture medium containing MTT and various concentrations of the test compound.

Commercial Availability

Commercial kits containing solutions of MTT and a solubilization reagent as well as MTT reagent powder are available from several vendors. For example:

- CellTiter 96® Non-Radioactive Cell Proliferation Assay. Promega Corporation Cat.# G4000,
- Cell Growth Determination Kit, MTT based. Sigma-Aldrich Cat.# CGD1-1KT, and
- MTT Cell Growth Assay Kit. Millipore Cat.# CT02.
- Thiazolyl Blue Tetrazolium Bromide (MTT Powder). Sigma-Aldrich Cat.# M2128.

The concentration of the MTT solution and the nature of the solubilization reagent differ among various vendors. The amount of formazan signal generated will depend on variety of parameters including the cell type, number of cells per well, culture medium, etc. Although the commercially available kits are broadly applicable to a large number of cell types and assay conditions, the concentration of the MTT and the type of solubilization solution may need to be adjusted for optimal performance.

Reagent Preparation

MTT Solution

1. Dissolve MTT in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) to 5 mg/ml.
2. Filter-sterilize the MTT solution through a 0.2 µM filter into a sterile, light protected container.
3. Store the MTT solution, protected from light, at 4°C for frequent use or at -20°C for long term storage.

Solubilization Solution

1. Choose appropriate solvent resistant container and work in a ventilated fume hood.
2. Prepare 40% (vol/vol) dimethylformamide (DMF) in 2% (vol/vol) glacial acetic acid.
3. Add 16% (wt/vol) sodium dodecyl sulfate (SDS) and dissolve.
4. Adjust to pH = 4.7
5. Store at room temperature to avoid precipitation of SDS. If a precipitate forms, warm to 37°C and mix to solubilize SDS.

MTT Assay Protocol

1. Prepare cells and test compounds in 96-well plates containing a final volume of 100 µl/well.
2. Incubate for desired period of exposure.
3. Add 10 µl MTT Solution per well to achieve a final concentration of 0.45 mg/ml.
4. Incubate 1 to 4 hours at 37°C.
5. Add 100 µl Solubilization solution to each well to dissolve formazan crystals.

6. Mix to ensure complete solubilization.
7. Record absorbance at 570 nm.

MTS Tetrazolium Assay Concept

More recently developed tetrazolium reagents can be reduced by viable cells to generate formazan products that are directly soluble in cell culture medium. Tetrazolium compounds fitting this category include MTS, XTT, and the WST series (18-23). These improved tetrazolium reagents eliminate a liquid handling step during the assay procedure because a second addition of reagent to the assay plate is not needed to solubilize formazan precipitates, thus making the protocols more convenient. The negative charge of the formazan products that contribute to solubility in cell culture medium are thought to limit cell permeability of the tetrazolium (24). This set of tetrazolium reagents is used in combination with intermediate electron acceptor reagents such as phenazine methyl sulfate (PMS) or phenazine ethyl sulfate (PES) which can penetrate viable cells, become reduced in the cytoplasm or at the cell surface and exit the cells where they can convert the tetrazolium to the soluble formazan product (25). The general reaction scheme for this class of tetrazolium reagents is shown in Figure 6.

In general, this class of tetrazolium compounds is prepared at 1 to 2mg/ml concentration because they are not as soluble as MTT. The type and concentration of the intermediate electron acceptor used varies among commercially available reagents and in many products the identity of the intermediate electron acceptor is not disclosed. Because of the potential toxic nature of the intermediate electron acceptors, optimization may be advisable for different cell types and individual assay conditions. There may be a narrow range of concentrations of intermediate electron acceptor that result in optimal performance.

Commercial Availability

Commercial kits containing solutions of MTS, XTT, and WST-1 and an intermediate electron acceptor reagent are available from several vendors. For example:

- CellTiter 96® AQueous One Solution Cell Proliferation Assay. Promega Corporation Cat.# G3580,
- In Vitro Toxicology Assay Kit, XTT based. Sigma-Aldrich Cat.# TOX2-1KT,
- Cell Counting Kit-8 (WST-8 based). Dojindo Molecular Technologies, Inc. Cat.# CK04-01,
- MTS Reagent Powder. Promega Corporation Cat.# G1111,
- XTT sodium salt. Sigma-Aldrich Cat.# X4626.

Reagent Preparation

MTS Solution (containing PES)

1. Dissolve MTS powder in DPBS to 2 mg/ml to produce a clear golden-yellow solution.
2. Dissolve PES powder in MTS solution to 0.21 mg/ml.

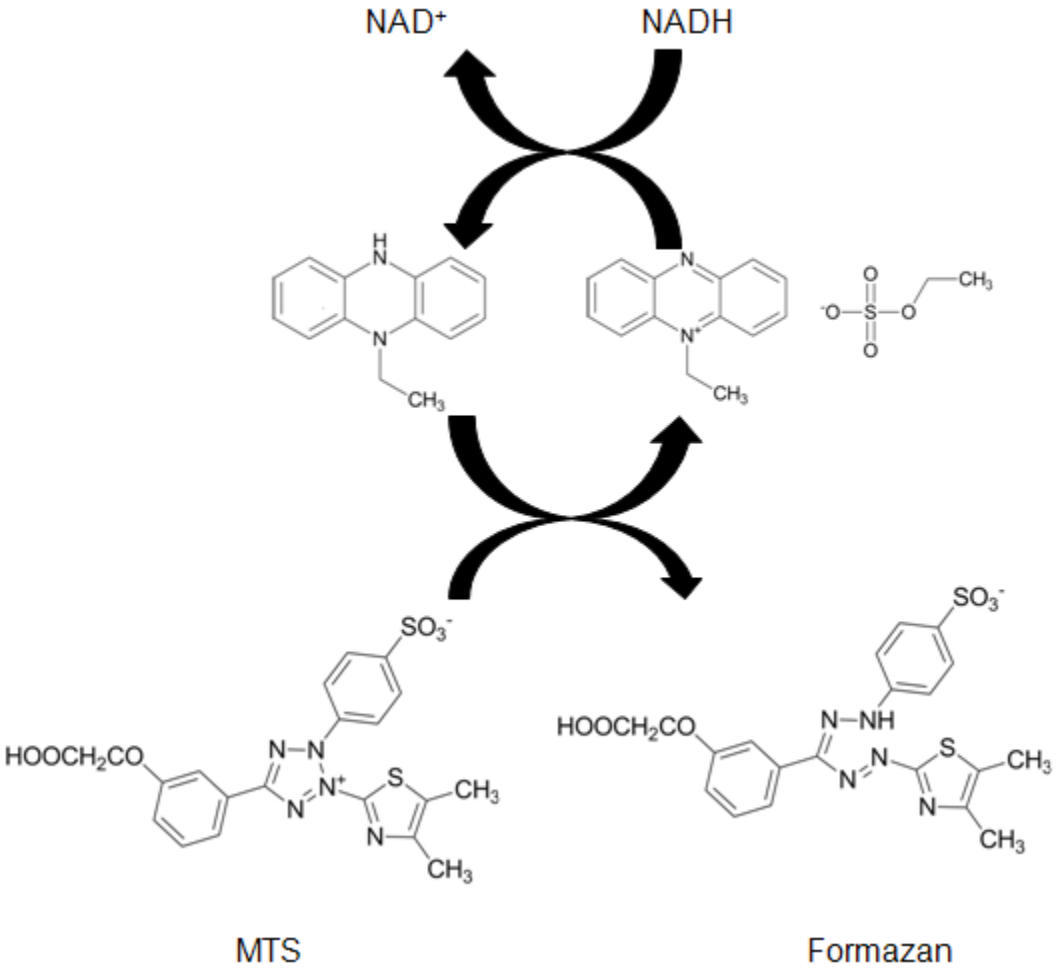

Figure 6: Intermediate electron acceptor pheazine ethyl sylfate (PES) transfers electron from NADH in the cytoplasm to reduce MTS in the culture medium into an aqueous soluble formazan.

3. Adjust to pH 6.0 to 6.5 using 1N HCl.
4. Filter-sterilize through a 0.2 µm filter into a sterile, light protected container.
5. Store the MTS solution containing PES protected from light at 4°C for frequent use or at -20°C for long term storage.

MTS Assay Protocol

1. Prepare cells and test compounds in 96-well plates containing a final volume of 100 µl/well. An optional set of wells can be prepared with medium only for background subtraction.
2. Incubate for desired period of exposure.
3. Add 20 µl MTS solution containing PES to each well (final concentration of MTS will be 0.33 mg/ml).

4. Incubate 1 to 4 hours at 37°C.
5. Record absorbance at 490 nm.

One of the advantages of the tetrazolium assays that produce an aqueous soluble formazan is that absorbance can be recorded form the assay plates periodically during early stages of incubation. Multiple readings may assist during assay development; but caution should be taken to return the plates to the incubator between readings to maintain a nearly constant environment. Extended incubations with the tetrazolium reagent beyond four hours should be avoided.

Whereas the background (culture medium and tetrazolium without cells) absorbance at 570 nm for an MTT assay may be 0.05, in general the background absorbance for the class of tetrazolium reagents is usually somewhat higher, in the range of 0.3 absorbance units and can depend on the type of culture medium and pH.

Resazurin Reduction Assay Concept

Resazurin is a cell permeable redox indicator that can be used to monitor viable cell number with protocols similar to those utilizing the tetrazolium compounds (26). Resazurin can be dissolved in physiological buffers (resulting in a deep blue colored solution) and added directly to cells in culture in a homogeneous format. Viable cells with active metabolism can reduce resazurin into the resorufin product which is pink and fluorescent (Figure 7).

Addition of an intermediate electron acceptor is not required for cellular resazurin reduction to occur, but it may accelerate signal generation. The quantity of resorufin produced is proportional to the number of viable cells which can be quantified using a microplate fluorometer equipped with a 560 nm excitation / 590 nm emission filter set. Resorufin also can be quantified by measuring a change in absorbance; however, absorbance detection is not often used because it is far less sensitive than measuring fluorescence. The resazurin reduction assay is slightly more sensitive than tetrazolium reduction assays and there are numerous reports using the resazurin reduction assay in a miniaturized format for HTS applications (27).

The incubation period required to generate an adequate fluorescent signal above background is usually 1to 4 hours and is dependent on the metabolic activity of the particular cell type, the cell density per well, and other assay conditions including the type of culture medium. The incubation period should be optimized and kept short enough to avoid reagent toxicity but long enough to provide adequate sensitivity.

The major advantages of the resazurin reduction assay are that it is relatively inexpensive, it uses a homogeneous format, and it is more sensitive that tetrazolium assays. In addition, resazurin assays can be multiplexed with other methods such as measuring caspase activity to gather more information about the mechanism leading to cytotoxicity (28 and Figure 8).

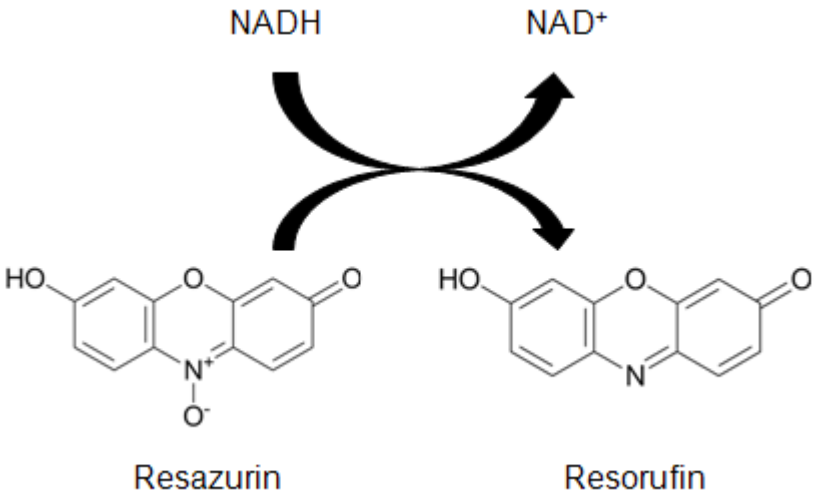

Figure 7: Structure of resazurin substrate and the pink fluorescent resorufin product resulting from reduction in viable cells.

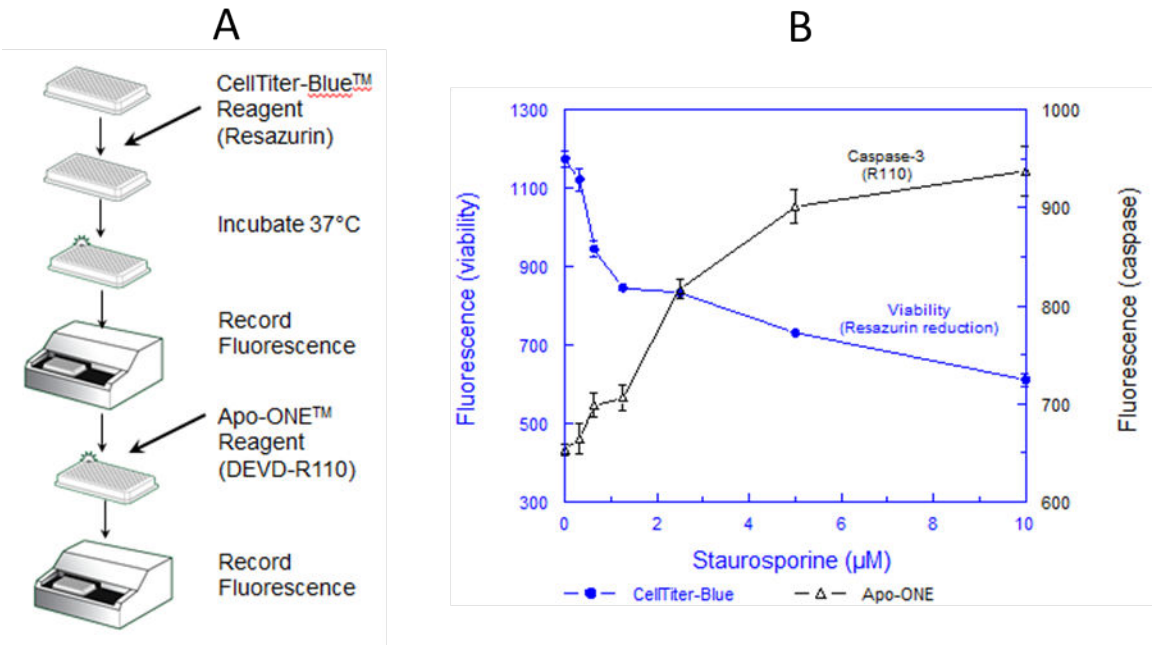

Figure 8: Panel A shows the steps of the sequential multiplex of a resazurin assay to measure viable cell number and a fluorometric caspase 3-assay to detect a marker of apoptosis. Panel B shows the results of treating PC3 (human prostate) cells with a range of concentrations of staurosporine for 20 hours. The resorufin (560/590 nm) and R110 fluorescence were captured at different wavelengths from the same sample as well.

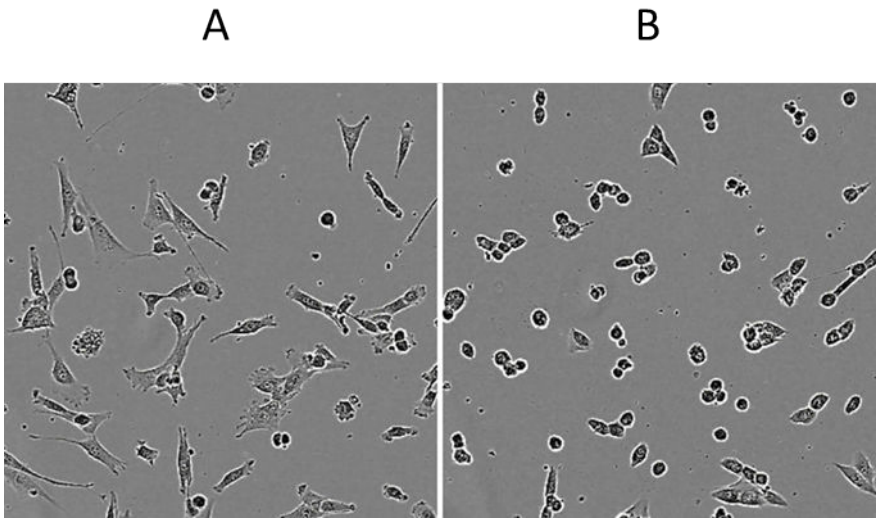

Figure 9: Change in NIH3T3 cell morphology after exposure to resazurin. Panel A shows a field of cells photographed immediately after addition of the resazurin solution. Panel B shows the same field of cells photographed after 4 hours of exposure to resazurin. Panel B shows a change in cell morphology. Images were captured using Incucyte from Essen Biosciences.

Multiplexing may require a sequential protocol to avoid color quenching by resazurin or direct chemical interference. For the multiplex example shown in Figure 8, resorufin fluorescence must be recorded first, followed by addition of the caspase reagent which contains detergent to lyse cells and reducing compounds to convert remaining resazurin and reduce interference with collecting the second fluorescent signal.

The disadvantages of the resazurin include the possibility of fluorescent interference from compounds being tested and the often overlooked direct toxic effects on the cells (Figure 9).

Some protocols describe exposing cells to resazurin for several hours or even days; however, in some systems, changes in cell morphology can be observed after only a few hours of exposure suggesting interference with normal cell function (29). It is possible that exposure of cells to resazurin depletes reduced forms of nucleotides resulting in cytotoxic effects. Exposure of cells to resazurin is known to reduce the amount of ATP measured as a marker of cell viability. Figure 10 shows a decrease in ATP content of HepG2 cells exposed to resazurin for 4 and 24 hours.

Commercial Availability

Commercial kits containing solutions of resazurin as well as resazurin powder are available from several vendors. For example:

- CellTiter-Blue® Cell Viability Assay. Promega Corporation Cat.# G8081,
- In Vitro Toxicology Assay Kit, Resazurin based. Sigma-Aldrich Cat.# TOX8-1KT,

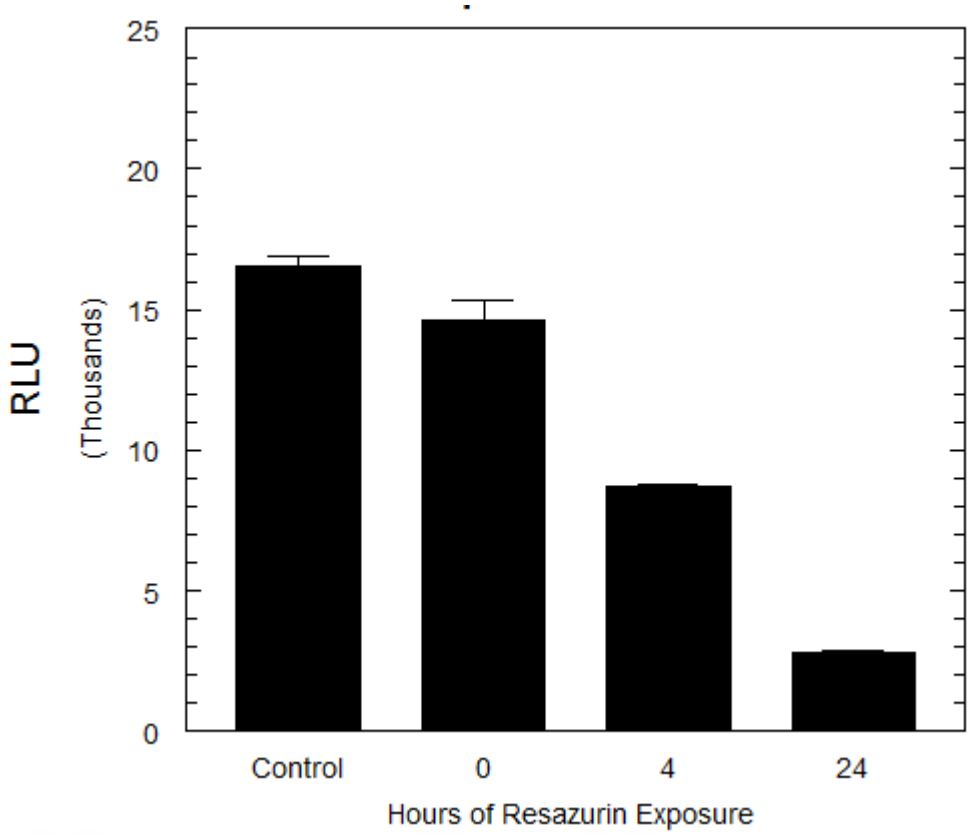

Figure 10: Viability (ATP content) of HepG2 cells exposed to resazurin for 0, 4, and 24 hours. Control wells did not contain resazurin. Zero hour wells contained resazurin and show quenching of luminescent signal following addition of the deeply blue colored resazurin reagent. ATP content was measured using the CellTiter-Glo® Assay.

- alamarBlue®—Rapid & Accurate Cell Health Indicator. Life Technologies, Inc. Cat.# DAL1100
- alamarBlue® AbD Serotech Cat.# BUF012B
- Resazurin sodium salt. Sigma-Aldrich Cat.# R7017-1G

Resazurin powder is readily available from chemical vendors; however, the resazurin dye content (% purity) and contamination with resorufin can lead to variability in assay results and the need to perform validation of each lot of reagent powder. Viability assay kits containing performance verified resazurin as the primary ingredient are available from different vendors; but the resazurin concentration and additional ingredients vary. The alamarBlue patent US 5,501,959 describes the use of poising agents to maintain the redox potential of the growth medium and prevent reduction of resazurin resulting in background signal (30). Preferred poising agents described include ferricyanide and ferrocyanide as well as methylene blue which can also serve as a redox indicator. The potential for undesired effects of additional ingredients in the proprietary alamarBlue formulation and the demonstrated performance equivalence of less complex formulations

of highly purified resazurin in balanced saline solution should be considered when choosing an assay reagent.

Reagent Preparation

1. Dissolve high purity resazurin in DPBS (pH 7.4) to 0.15 mg/ml.
2. Filter-sterilize the resazurin solution through a 0.2 µm filter into a sterile, light protected container.
3. Store the resazurin solution protected from light at 4°C for frequent use or at -20°C for long term storage.

Resazurin Assay Protocol

1. Prepare cells and test compounds in opaque-walled 96-well plates containing a final volume of 100 µl/well. An optional set of wells can be prepared with medium only for background subtraction and instrument gain adjustment.
2. Incubate for desired period of exposure.
3. Add 20 µl resazurin solution to each well.
4. Incubate 1 to 4 hours at 37°C.
5. Record fluorescence using a 560 nm excitation / 590 nm emission filter set.

A general disadvantage of both the tetrazolium and resazurin reduction assay protocols is the requirement to incubate the substrate with viable cells at 37°C for an adequate period of time to generate a signal. Incubation of the tetrazolium or resazurin reagents with viable cells increases the possibility of artifacts resulting from chemical interactions among the assay chemistry, the compounds being tested, and the biochemistry of the cell. Incubation also introduces an extra plate handling step that is not required for the ATP assay protocol described later. Extra plate manipulation steps increase the possibility of errors and are not desirable for automated assays for HTS.

Protease Viability Marker Assay Concept

Measurement of a conserved and constitutive protease activity within live cells has been shown to serve as a marker of cell viability. A cell permeable fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) has recently been developed to selectively detect protease activity that is restricted to viable cells (31). The GF-AFC substrate can penetrate live cells where cytoplasmic aminopeptidase activity removes the gly and phe amino acids to release aminofluorocoumarin (AFC) and generate a fluorescent signal proportional to the number of viable cells (Figure 11).

As soon as the cells die, this protease activity rapidly disappears, thus making this protease activity a selective marker of the viable cell population. This assay approach is available as a commercial product from Promega Corporation (32). The components of the product include: GF-AFC 100mM in DMSO and an Assay Buffer for dilution of the substrate. The signal generated from the protease assay approach has been shown to correlate well with other established methods of determining cell viability such as an ATP assay (Figure 12).

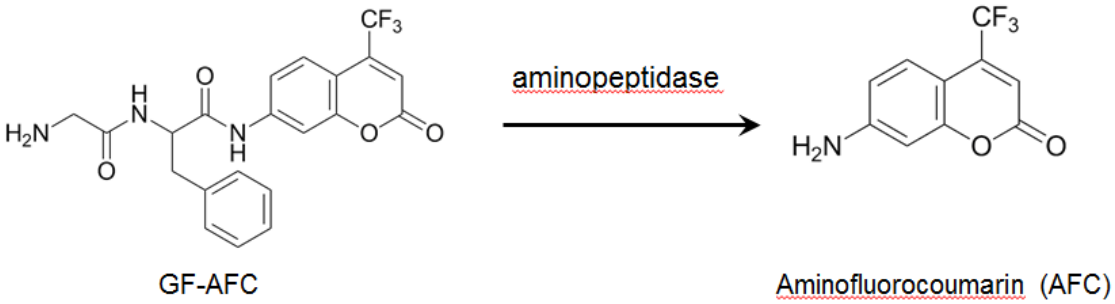

Figure 11: Cell permeable glycylphenylalanyl-aminofluoroumarin (GF-AFC) substrate is converted by cytoplasmic aminopeptidase activity to generate fluorescent aminofluorocoumarin (AFC).

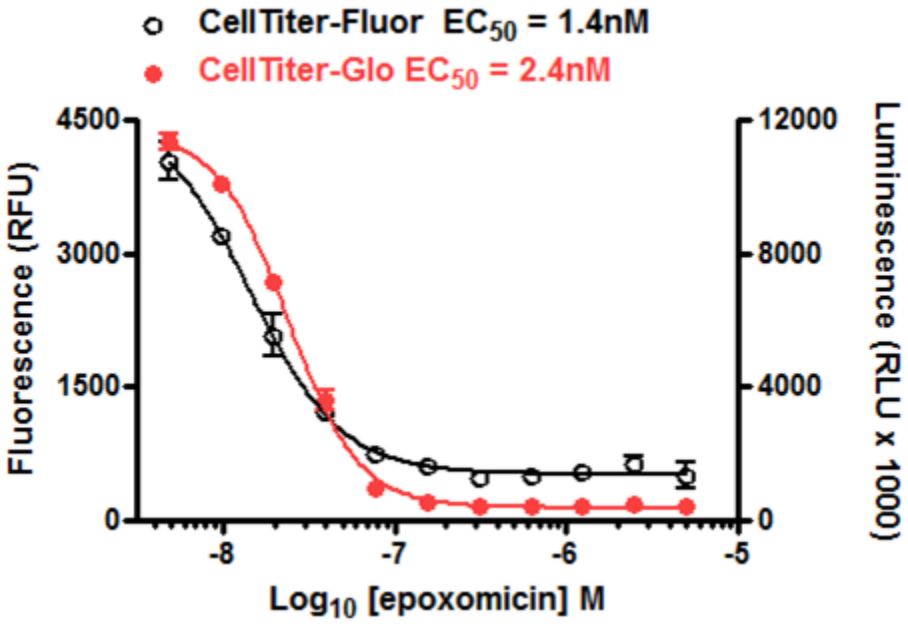

Figure 12: DU-145 cells treated with various concentrations of epoxomicin for 48 hours and assayed using GF-AFC reagent (CellTiter-Fluor™, open circles) and ATP detection (CellTiter-Glo® Assay, solid red circles). The similar EC_{50} values demonstrate good correlation between different methods to estimate viable cells.

One of the advantages of the GF-AFC substrate is that it is relatively non-toxic to cells in culture (Figure 13).

In addition, long term exposure of the GF-AFC substrate to cells results in little change in viability measured using ATP as a marker. This is in direct contrast to the effects of exposing cells to tetrazolium or resazurin redox indicators which have been demonstrated

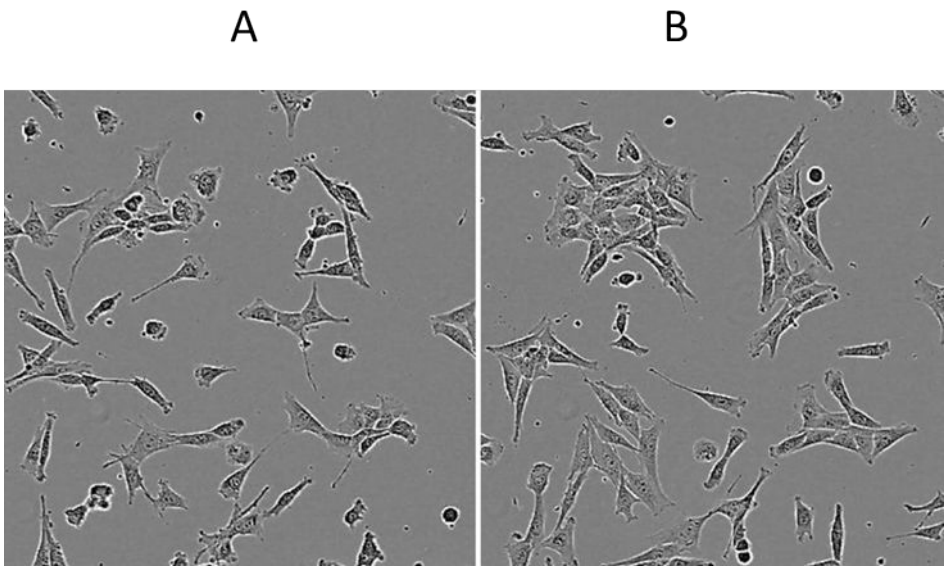

Figure 13: Morphology of NIH3T3 cells during exposure to GF-AFC reagent. Panel A shows a field of cells photographed immediately after additional of the GF-AFC reagent. Panel B shows the same cells photographed after 4 hours of exposure to GF-AFC. Panel B shows a little change in the cell morphology compared to the substantial changes and obvious toxicity shown for MTT and resazurin in the figures above. Images were captured using the IncuCyte™ FLR from Essen Biosciences.

to be toxic to cells as described above. The non-toxic nature of the GF-AFC substrate makes it an ideal candidate for multiplexing with other assay technologies using a sequential assay protocol. After recording fluorescence data from the live cell protease assay, the population of cells remains viable and can be used for subsequent assays as long as the fluorescent signal from AFC does not interfere. This property enables "on-the-fly" detection and follow-up of cytotoxic hits during screening campaigns. Wells containing hits can be subjected to an orthogonal method to detect viable cell number or an alternate assay method to detect the mechanism leading to cell death. Figure 14 shows an example of multiplexing the live cell protease marker and a luminescent caspase assay to detect apoptosis. In this example, the decrease in viability corresponds to an increase in caspase activity suggesting the mode of cell death is via apoptosis. An advantage of measuring this protease as a viability marker is that in general, the incubation time required to get an adequate signal is much shorter (30 min to 1 hour), compared to 1 to 4 hours required for the tetrazolium assays.

GF-AFC Reagent Preparation

1. Thaw the GF-AFC substrate and Assay Buffer components from the CellTiter-Fluor™ Cell Viability Assay kit following the detailed procedure in the Technical Bulletin #371 (32).
2. Transfer 10 µl of the GF-AFC Substrate into 10 ml of the Assay Buffer to prepare a 2X Reagent. Note: For multiplexing applications where total sample volume is a

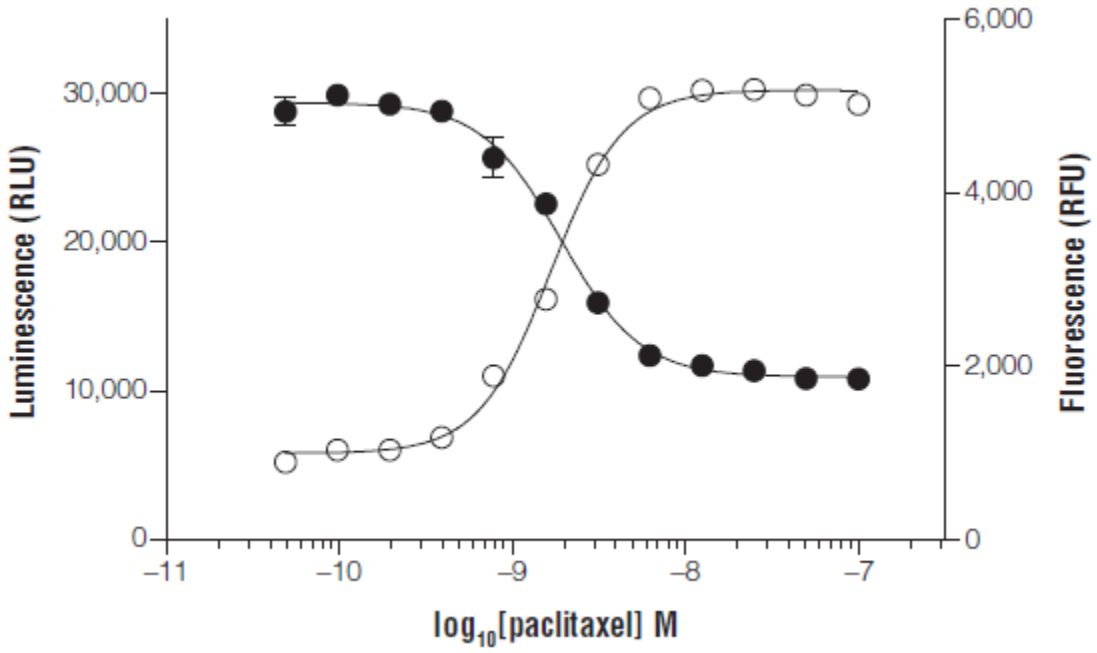

Figure 14: (Modified Figure 5 from TB371 32). Multiplex measurement of the live cell protease marker using GF-AFC (CellTiter-Fluor™ Assay) followed by measurement of caspase activity (Caspase-Glo® 3/7 Assay). The GF-AFC substrate was added to wells containing 10,000 cells/well, incubated for 30 minutes at 37°C and fluorescence (380–400nmEx/505nmEm) measured to estimate viable cell number. Following collection of the fluorescence data, caspase activity was measured using the luminogenic Caspase-Glo® 3/7 Reagent. Luminescence measured after 30-minutes incubation (Caspase-Glo TB323 33).

concern, a 10X Reagent can be prepared by adding 10 µl GF-AFC Substrate to 2 ml of Assay Buffer.

3. Mix by vortexing the contents until the GF-AFC substrate is thoroughly dissolved.

Storage: Store the CellTiter-Fluor™ Cell Viability Assay components at –20°C. The diluted CellTiter-Fluor™ Viability Reagent should be used within 24 hours if stored at room temperature. Unused GF-AFC Substrate and Assay Buffer can be stored at 4°C for up to 7 days with no appreciable loss of activity.

Live Cell Protease Assay Protocol

1. Set up opaque-walled 96-well assay plates containing cells in culture medium at desired density. An optional set of wells can be prepared with medium only for background subtraction and instrument gain adjustment.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100 µl in each well (25 µl for a 384-well plate).
3. Culture cells for the desired test exposure period.
4. Add CellTiter-Fluor™ Reagent in an equal volume (100 µl per well) to all wells, mix briefly by orbital shaking, then 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, and be sure to shield plates from ambient light.

5. Measure resulting fluorescence using a fluorometer (380–400 nm Ex/505 nm Em).

ATP Assay Concept

The measurement of ATP using firefly luciferase is the most commonly applied method for estimating the number of viable cells in HTS applications. Data from several example HTS assays using ATP assays are publically available on Pubchem (34). ATP has been widely accepted as a valid marker of viable cells. When cells lose membrane integrity, they lose the ability to synthesize ATP and endogenous ATPases rapidly deplete any remaining ATP from the cytoplasm. Although luciferase has been used to measure ATP for decades, recent advances in assay design have resulted in a single reagent addition homogeneous protocol that results in a luminescent signal that glows for hours. The most significant technological advancement was made under the direction of Keith Wood at Promega Corporation where directed evolution was used to select for stable molecules and generate improved versions of luciferase (35). The stable version of luciferase was the enabling technology that led to development of robust assays for HTS that can withstand harsh cell lysis conditions and are more resistant to luciferase inhibitors found in libraries of small molecules (36).

The ATP detection reagent contains detergent to lyse the cells, ATPase inhibitors to stabilize the ATP that is released from the lysed cells, luciferin as a substrate, and the stable form of luciferase to catalyze the reaction that generates photons of light. A simplified reaction scheme is shown in Figure 15.

The ATP assay is the fastest cell viability assay to use, the most sensitive, and is less prone to artifacts than other viability assay methods. The luminescent signal reaches a steady state and stabilizes within 10 minutes after addition of reagent and typically glows with a half-life greater than 5 hours. The ATP assay has the advantage that you do not have to rely on an incubation step with a population of viable cells to convert a substrate (such a tetrazolium or resazurin) into a colored compound. This also eliminates a plate handling step because you do not have to return cells to the incubator to generate signal.

The ATP assay chemistry can typically detect fewer than 10 cells per well and has been used widely in 1536-well format. The ATP assay sensitivity is usually limited by reproducibility of pipetting replicate samples rather than a result of the assay chemistry.

Commercial Availability

Commercial kits containing reagents to measure ATP are available from several vendors. For example:

- CellTiter-Glo® Luminescent Cell Viability Assay. Promega Corporation Cat.# G7570
- ATPLite™ 1 step, Perkin Elmer Cat.# 6016731,

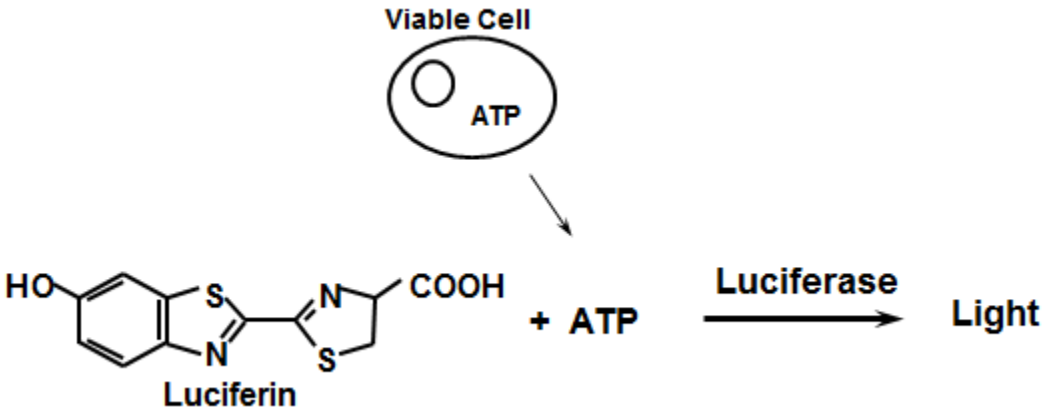

Figure 15: Simplified reaction scheme showing ATP and luciferin as substrates for luciferase to generate light.

- Adenosine 5′-triphosphate (ATP) bioluminescent somatic cell assay kit. Sigma-Aldrich Cat.# FLASC-1KT.

The most common version of the CellTiter-Glo® Assay kit contains a lyophilized CellTiter-Glo® Substrate and the CellTiter-Glo® Buffer which are both stored at –20°C; however, a bulk frozen liquid version of CellTiter-Glo® Assay also is available which eliminates the step of reconstituting the lyophilized Substrate. For more detailed information, refer to Promega Technical Bulletin #288 (37).

ATP Assay Reagent Preparation

1. Thaw the CellTiter-Glo® Buffer and CellTiter-Glo® Substrate and equilibrate to room temperature prior to use. For convenience the CellTiter-Glo® Buffer may be thawed and stored at room temperature for up to 48 hours prior to use.
2. Transfer the appropriate volume (10ml for Cat.# G7570) of CellTiter-Glo® Buffer into the amber bottle containing CellTiter-Glo® Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the CellTiter-Glo® Reagent.
3. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution. The CellTiter-Glo® Substrate should go into solution easily in less than 1 minute.

ATP Assay Protocol

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

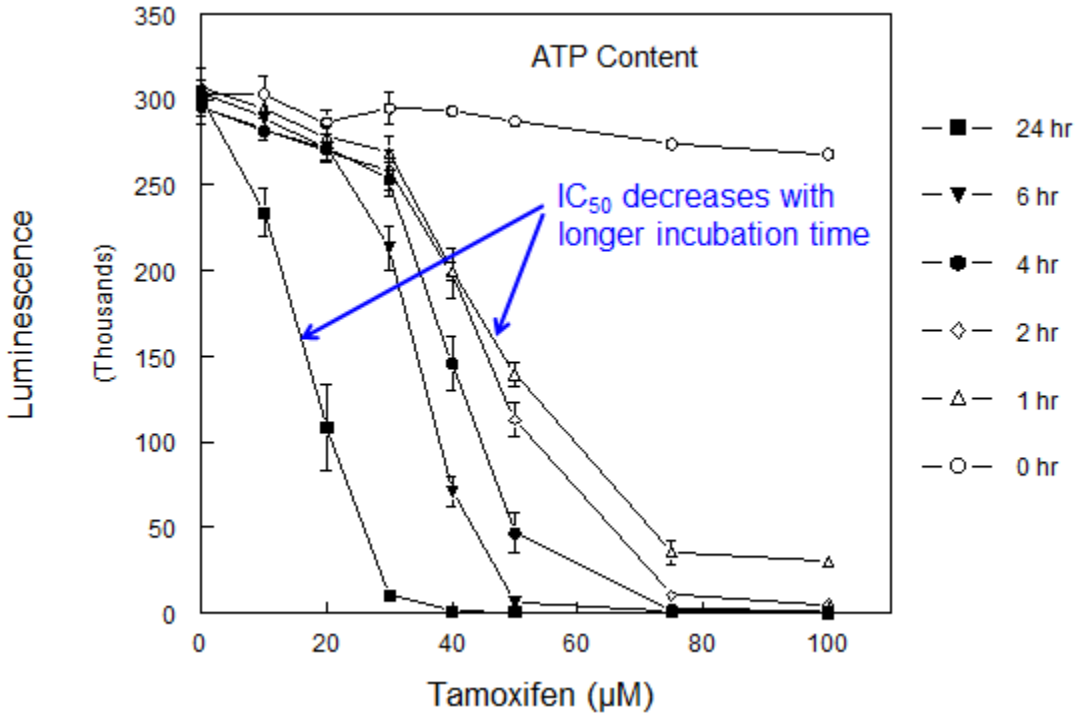

Figure 16: HepG2 cells (25,000 cells in 100ul medium/well) were cultured overnight in an opaque-walled 96 well plate then treated with 0-100uM tamoxifen in DMSO (final concentration of 0.2% DMSO) for various times. ATP content was measured by adding 100ul CellTiter-Glo® Reagent and recording luminescence after a 10min equilibration period. Data shown represent the mean +/- SD (n = 3). Modified from Figure 1 from Assay & Drug Devel Tech 2(1): 51, 2004 (38).

4. Equilibrate plates to ambient temperature for 30 min to ensure uniform temperature across plate during luminescent assay.
5. Add CellTiter-Glo® Reagent in an equal volume (100 µl per well for 96-well plates or 25 µl per well for 384-well plates) to all wells.
6. Mix contents for 2 minutes on an orbital shaker to induce cell lysis.
7. Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal. Note: Uneven luminescent signal within standard plates can be caused by temperature gradients, uneven seeding of cells or edge effects in multiwall plates.
8. Record luminescence.

Figure 16 shows the results of an example assay characterization experiment to determine the appropriate time to record viability data for a cell-based assay.

Real-Time Assay for Viable Cells

A recently developed approach for measuring viable cell number in “real time” utilizes an engineered luciferase derived from a marine shrimp and a small molecule pro-substrate

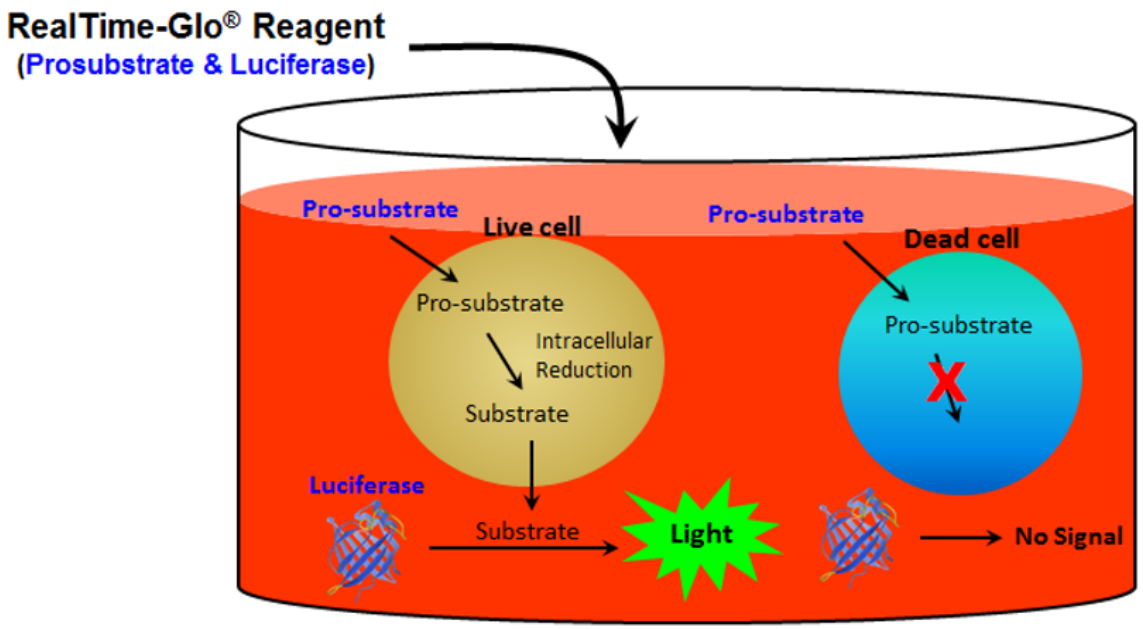

Figure 17: The real-time reagent components include a cell permeable pro-substrate and an engineered stable form of a shrimp-derived luciferase. The reagent components are added directly to cells in culture. Viable cells with an active metabolism reduce the pro-substrate to create a substrate for luciferase that generates light. Dead cells lacking metabolic activity do not generate luciferase substrate and thus do not contribute a luminescent signal.

(39). The pro-substrate and luciferase are added directly to the culture medium as a reagent. The pro-substrate is not a substrate for luciferase. Viable cells with an active metabolism reduce the pro-substrate into a substrate which diffuses into the culture medium where it is used by luciferase to generate a luminescent signal. Figure 17 shows an illustration of the assay concept.

The reagent is well tolerated by cells and is stable in complete cell culture medium at 37°C for at least 72 hours which enables measurements from the same sample for days without replenishing the pro-substrate. The assay can be performed in two formats: continuous-read or endpoint measurement. In the continuous-read format, the luminescent signal can be repeatedly recorded from the same sample wells over an extended period to measure the number of viable cells in "real time".

Figure 18 shows example results from a toxin dose-response assay using the real time format for 3 days. The cells in the vehicle control and the lowest concentrations of thapsigargin continue to grow and show an increase in luminescence over the three day period. Samples of cells treated with the highest concentrations of thapsigargin show a decrease in luminescence over time as the cells die.

For convenience, in the continuous read format, the reagents can be added to the cell suspension prior to dispensing into assay plates. This approach eliminates a pipetting step and a potential source of variability during delivery of assay reagent into samples. In the

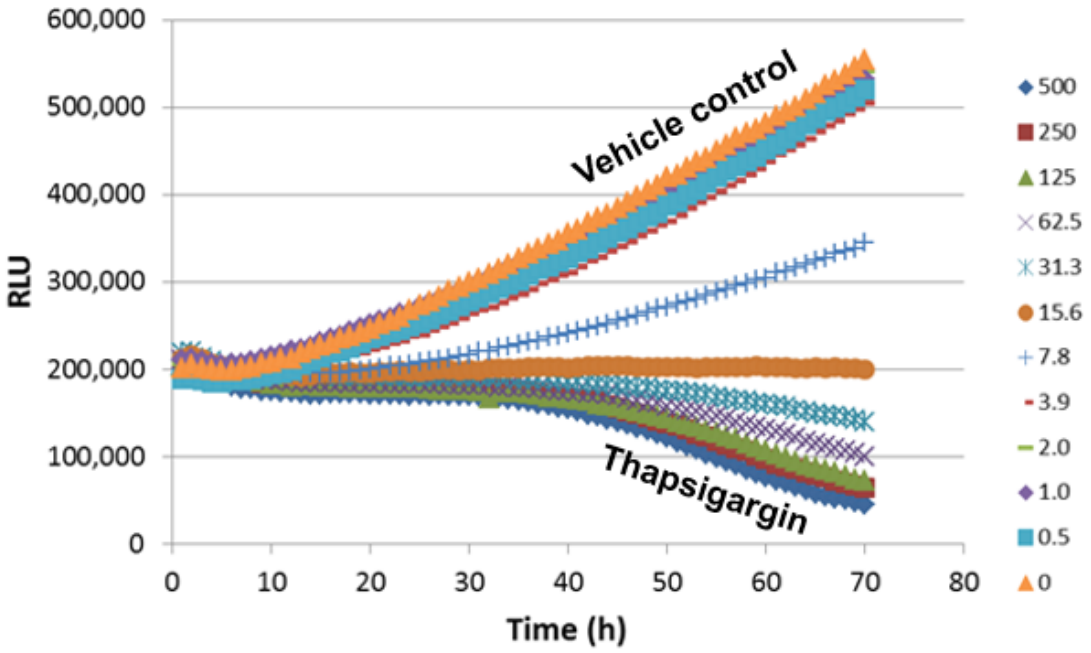

Figure 18: A549 cells (500/well) were plated in 40µL medium containing 2x RealTime-Glo™ reagents. A thapsigargin titration was prepared in medium at 2X concentrations and added to the plate at an equal volume. The final concentrations of thapsigargin ranged from 500nM - 0.5nM. The vehicle control was 0.1% DMSO. Luminescence was monitored every hour for 72 hours using a Tecan Infinite® 200 Multimode Reader with Gas Control Module (37°C and 5% CO_2).

endpoint format, the reagent can be added to cells at any time during the experimental period. A steady state develops between viable cells reducing pro-substrate to convert it into the luciferase substrate, the appearance of the substrate in the culture medium and the luciferase enzymatic reaction using the substrate to generate light. For the endpoint format, luminescence can typically be recorded within 10 minutes to an hour after adding reagent to the cells, depending on assay conditions.

The substrate produced by viable cells is used rapidly by the luciferase, thus the luminescent signal diminishes soon after cell death. Figure 19 illustrates the decrease in luminescent signal following addition of digitonin to kill the cells.

The rapid decrease in luminescent signal following cell death enables multiplexing of the real time viability assay with other luminescent assays that contain a lysis step that will kill cells. The decrease in luminescence from the real time viability assay following cell death is important to eliminate interference with subsequent luminescent assays that use firefly luciferase.

Multiplexing with the Real Time Viability Assay

Because the real time reagent does not contain detergent (i.e. is non-lytic) and is well tolerated by most cell types, after recording viability data, the remaining sample of cells

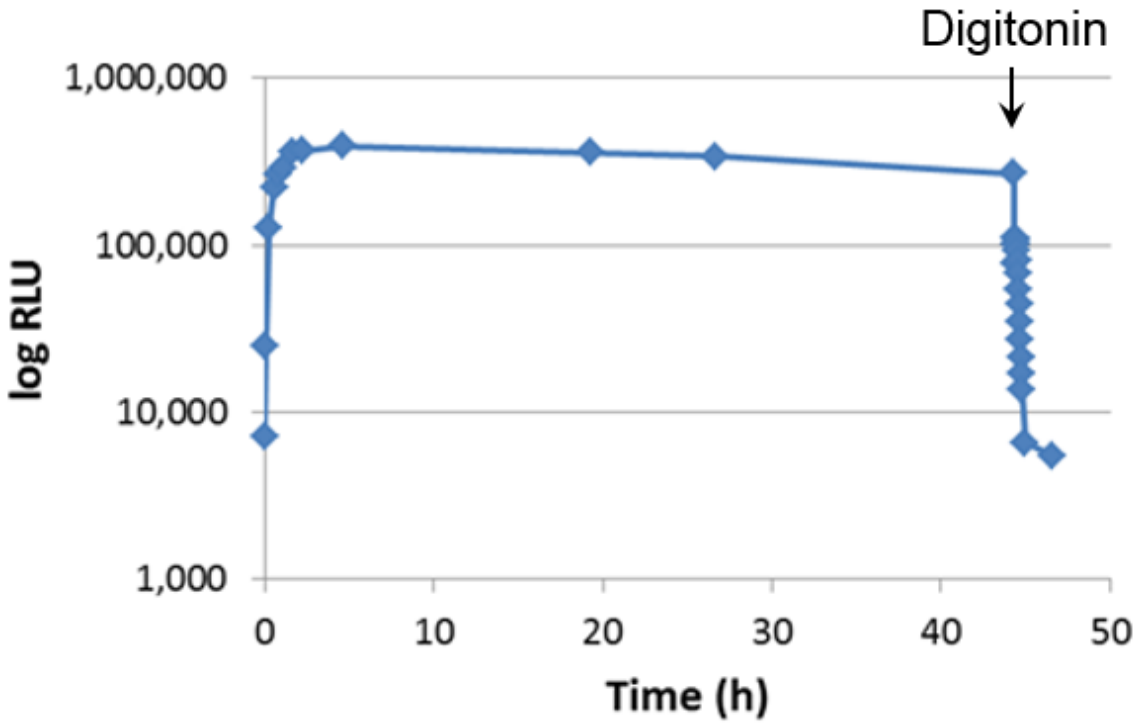

Figure 19: iCell cardiomyocytes (Cellular Dynamics, Inc.) were plated and grown in medium containing RealTime-Glo™ reagents (pro-substrate and NanoLuc luciferase) in a 37°C/5% CO_2 humidified incubator. At various time points, the luminescence signal was monitored on a Tecan M1000Pro plate reader. After 2 days, digitonin was added to a final concentration of 200 µg/ml. The luminescence was read continually, starting immediately after digitonin addition.

can be used for many downstream applications. Multiplexing can be achieved with a variety of other assay chemistries including: most assays with a fluorescent detection method, the luminescent ATP assay as an orthogonal approach to confirm viability data with more than one method, a luminescent caspase-3/7 assay to measure apoptosis, firefly reporter assays to monitor gene expression, and extraction of RNA that can be used to monitor gene expression.

Figure 20 illustrates an example showing the effect of a dose-response of a proteasome inhibitor on the viability of cells measured at different times from the same samples using the real time viability assay followed by multiplex measurement of ATP as an orthogonal method to demonstrate concordance between the two viability assays. The sequential multiplexing example shows results from recording luminescence from a shrimp-derived luciferase followed by recording luminescence from a firefly-derived luciferase. The ATP assay contains detergent to lyse cells to release ATP as well as luciferin and a stable form of luciferase necessary to measure ATP (see general description above in this chapter). The detergent lysis step stops the ability of cells to generate a substrate for the shrimp luciferase and thus diminishes the luminescent signal from the real time viability assay. This sequential combination of reagents makes it possible to record two luminescent

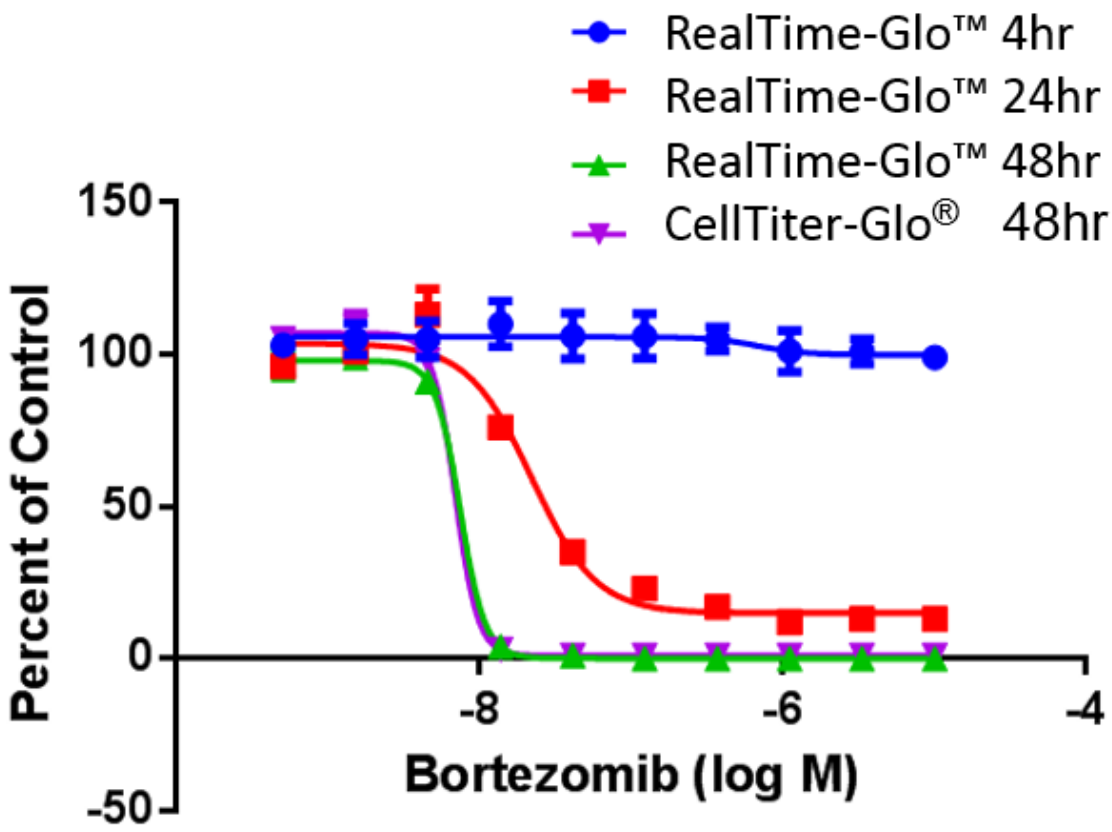

Figure 20: K562 cells in complete medium (RPMI medium supplemented with 10% FBS and Pen/Strep) containing the RealTime-Glo™ Reagent were seeded at 2500 cells in 50µl/well into 96 well opaque white plates. Bortezomib dilutions were prepared from DMSO stock as 2X final concentration in complete culture medium and 50µl were added to appropriate wells. The vehicle control was 0.6% DMSO in complete culture medium. Luminescence from the real time viability assay was recorded after 4, 24 and 48 hours incubation, then 100µl/well of the CellTiter-Glo® 2.0 Reagent was added to each well, the plate was stored at room temperature for 30 min to ensure cell lysis, then luminescence recorded. The values represent the mean ± SD of 4 replicates and were normalized to 100% assigned to the vehicle control for each assay.

signals from two different luciferases from the same sample. The 48 hour data from the real time assay approach agrees well with the 48 hour data from the ATP assay, demonstrating concordance between these two methods. Similar agreement between assays has been observed from the combination of the real time viability assay and a constitutive firefly reporter gene assay (not shown).

Figure 21 shows another example of multiplexing the real time viability assay using the shrimp luciferase and a caspase-3/7 assay using firefly luciferase. A gradual decrease in viability and increase in caspase-3/7 activity was observed over the first ~30 hours. The decrease in caspase-3/7 activity at the longer incubations times is likely due to secondary necrosis of the cells resulting in loss of activity of the caspase enzyme. This assay

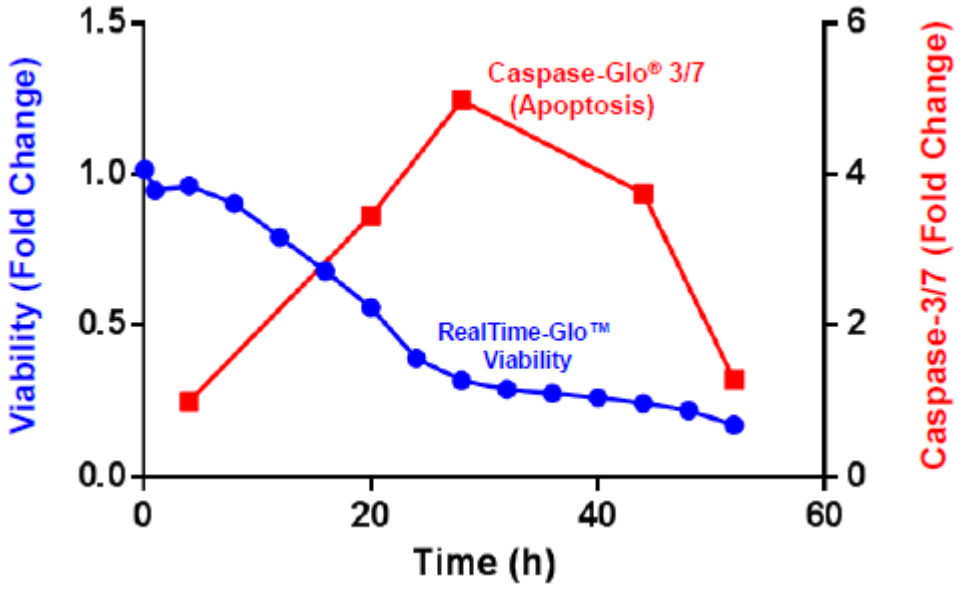

Figure 21: THP1 cells were grown in medium containing the RealTime-Glo™ Assay Reagent and treated with 1µM doxorubicin. Luminescence was recorded every 4 hours to monitor changes in cell viability. At selected times, Caspase-Glo® 3/7 Assay Reagent supplemented with a specific inhibitor for the shrimp luciferase was added to parallel wells. The plates were incubated at room temperature for 1 hour, then luminescence recorded from the firefly-derived luciferase in the caspase detection reagent.

combination exemplifies a special case where reagent chemical compatibility during multiplexing can be a problem. Caspase assay reagent formulations typically contain reducing agents (such as dithiothreitol) which can result in some chemical reduction of the pro-substrate into substrate. The substrate generated from chemical reduction of the pro-substrate can be used by the active shrimp luciferase and contribute to background luminescence. For those situations, the addition of a specific chemical inhibitor of the shrimp luciferase eliminates signal from that enzyme so the luminescence does not interfere with the signal from firefly luciferase used in a multiplexed secondary assay.

The real time viability assay enables monitoring for early cytotoxic events in populations of cells exposed to drugs. Analysis of RNA extracted from a population of cells that show the first signs of cell death (i.e. when most of the cells are still viable) can provide information about which stress response genes are expressed during experimental treatments. The real time viability assay reagent has been shown to have little effect on yield or integrity of RNA. That is in contrast to the ATP assay reagent which contains a high concentration of detergent to lyse cells resulting in poor recovery and loss of integrity of RNA. Figure 22 shows the results of extracting RNA from different sizes of individual 3D spheroids of HEK293 cells after measurement of cell viability using the real time assay reagent. RNA was extracted using either a manual or an automated method. For each method, the presence or absence of the real time reagent did not affect the recovery of RNA from spheroids. In addition, the RIN values (used as an indicator of the

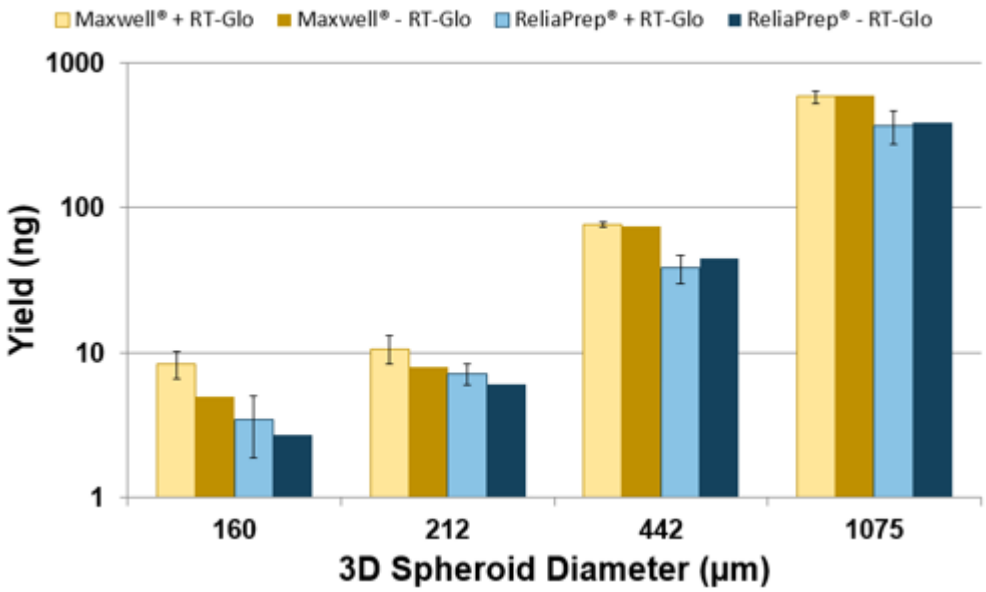

Figure 22: HEK293 spheroids of different sizes were prepared using the GravityPLUS™ Hanging Drop System from InSphero. RealTime-Glo™ MT Cell Viability Assay was used to measure viability of different sizes of HEK293 cell spheroids followed by RNA extraction of the same samples using a manual method (ReliaPrep™ RNA Tissue Miniprep System) and an automated method (Maxwell® 16 LEV simply RNA Tissue Kit). Each RNA extraction method was done in the presence and absence of RealTime-Glo™ Reagent for each of the different sizes of spheroid. Each bar represents the mean +/- SD of 3 spheroids.

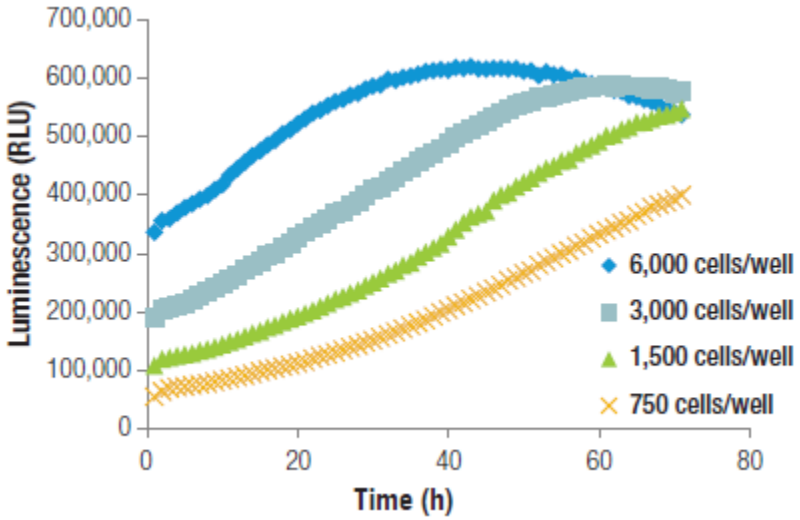

Figure 23: K562 cells were seeded at 750, 1500, 3000 or 6000 cells/well in a 384-well opaque white plate in 80µL of RPMI medium supplemented with 10% FBS and Pen/Strep that contained the RealTime-Glo™ Reagent. Luminescence was monitored every hour for 72 hours using a Tecan Infinite® 200 Multimode Reader with Gas Control Module (37°C and 5% CO_2).

integrity of the RNA) were in the excellent range (~8 to 9.5) and were not affected by the presence of the real time cell viability assay reagent.

A limitation of the real time assay format results from the eventual depletion of pro-substrate by metabolically active cells. In general, the luminescent signal generated correlates with the number of metabolically active viable cells; however, the length of time the luminescent signal will be linear with cell number will depend on the number of cells per well and their overall metabolic activity. Figure 23 shows luminescent signals recorded every hour for 72 hours from wells initially seeded with 750, 1500, 3000, or 6000 K562 cells/well in a 384-well plate. The signal from 750 and 1500 cells/well remain linear over the 3 day period, whereas the signal from higher cell numbers per well lose linearity after different times of incubation. It is recommended that the maximum incubation time to maintain linearity should be empirically determined for each cell type and seeding density.

Commercial Availability

Commercial kits containing the pro-substrate and the engineered shrimp-derived luciferase are available from Promega Corporation. RealTime-Glo™ MT Cell Viability Assay, Cat.# G9711 (100 reactions); G9712 (10x100 reactions); G9713 (1000 reactions)

Reagent Preparation and Real Time Viability Assay Protocol

The MT Cell Viability Substrate and the NanoLuc® Enzyme are both supplied at 1000X the final recommended concentration.

For continuous read mode:

1. Equilibrate the MT Cell Viability Substrate and the NanoLuc® Enzyme to 37°C.
2. Harvest cells and adjust to desired cell density to be used in the assay.
3. Add MT Cell Viability Substrate and the NanoLuc® Enzyme to the cell suspension.
4. Dispense cell suspension containing MT Cell Viability Substrate and the NanoLuc® Enzyme into white opaque walled multiwell plates suitable for luminescence measurements.
5. Add test compound and incubate for desired length of time.
6. Record luminescence.

For endpoint mode:

1. Harvest cells and adjust to desired cell density to be used in the assay.
2. Add test compound to cells and incubate for desired length of time.
3. Equilibrate the MT Cell Viability Substrate and the NanoLuc® Enzyme to 37°C.
4. Dilute MT Cell Viability Substrate and the NanoLuc® Enzyme in cell culture medium to form 2X RealTime-Glo™ Reagent.
5. Add an equal volume of 2X RealTime-Glo™ Reagent to cells.
6. Incubate at 37°C for 10-60 min.
7. Record luminescence.

Conclusion

There are a variety of assay technologies available that use standard plate readers to measure metabolic markers to estimate the number of viable cells in culture. Each cell viability assay has its own set of advantages and disadvantages. The ATP detection assay is by far the most sensitive, has fewer steps, is the fastest to perform, and has the least amount of interference, whereas the tetrazolium or resazurin reduction assays offer less expensive alternatives that may achieve adequate performance depending on experimental design. The fluorogenic cell permeable protease substrate is far less cytotoxic than the tetrazolium and resazurin compounds while enabling many possibilities for multiplexing other assays to serve as orthogonal or confirmatory methods. The recently developed cell viability assay, based on generating a substrate for the shrimp luciferase, provides the opportunity for capturing data repeatedly in real time and offers many possibilities for multiplexing with other assays. Regardless of the assay method chosen, the major factors critical for reproducibility and success include: 1) using a tightly controlled and consistent source of cells to set up experiments and 2) performing appropriate characterization of reagent concentration and incubation time for each experimental model system.

References

1. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Meth. 1983;65:55–63. PubMed PMID: 6606682.
2. Marshall NJ, Goodwin CJ, Holt SJ. A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. Growth Regul. 1995;5(2):69–84. PubMed PMID: 7627094.
3. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys. 1993;303(2):474–82. PubMed PMID: 8390225.
4. Berridge M., Tan A., McCoy K., Wang R. The Biochemical and Cellular Basis of Cell Proliferation Assays that Use Tetrazolium Salts. Biochemica. 1996;4:14–19.
5. Tada H, Shiho O, Kuroshima K, et al. An improved colorimetric assay for interleukin 2. J. Immunol. Methods. 1986;93:157–65. PubMed PMID: 3490518.
6. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods. 1989;119:203–210. PubMed PMID: 2470825.
7. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Meth. 1986;89:271–277. PubMed PMID: 3486233.
8. Plumb JA, Milroy R, Kaye SB. 1989. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on

chemosensitivity determined by a novel tetrazolium-based assay. Cancer Res 15;49(16):4435-40.
9. CellTiter 96® Non-Radioactive Cell Proliferation Assay Technical Bulletin #112. Online at [Available at: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/0/CellTiter%2096%20Non-Radioactive%20Cell%20Proliferation%20Assay%20Protocol.pdf]
10. Huyck L, Ampe C, Van Troys M. The XTT cell proliferation assay applied to cell layers embedded in three-dimensional matrix. Assay Drug Dev Tech. 2012;10(4): 382–392. PubMed PMID: 22574651.
11. Squatrito R, Connor J, Buller R. Comparison of a novel redox dye cell growth assay to the ATP bioluminescence assay. Gynecologic Oncology. 1995;58:101–105. PubMed PMID: 7789873.
12. Lü L, Zhang L, Wai MS, Yew DT, Xu J. Exocytosis of MTT formazan could exacerbate cell injury. Toxicol In Vitro. 2012;26(4):636–44. Epub 2012 Feb 28. PubMed PMID: 22401948.
13. Ulukaya E, Colakogullari M, Wood E J. Interference by anti-cancer chemotherapeutic agents in the MTT-tumor chemosensitivity assay. Chemotherapy. 2004;50(1):43–50. PubMed PMID: 15084806.
14. Chakrabarti R, Kundu S, Kumar S, Chakrabarti R. Vitamin A as an enzyme that catalyzes the reduction of MTT to formazan by vitamin C. J Cellular Biochem. 2000;80(1):133–138. PubMed PMID: 11029760.
15. Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry. 2002;47(4):236–42. PubMed PMID: 11933013.
16. Pagliacci M, Spinozzi F, Migliorati G, et al. Genistein inhibits tumour cell growth in vitro but enhances mitochondrial reduction of tetrazolium salts: a further pitfall in the use of the MTT assay for evaluating cell growth and survival. Eur J Cancer. 1993;29:1573–1577. PubMed PMID: 8217365.
17. Collier A, Pritsos C. The mitochondrial uncoupler dicumerol disrupts the MTT assay. Biochem Pharm. 2003;66:281–287. PubMed PMID: 12826270.
18. Cory A, Owen T, Barltrop J, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun. 1991;3(7):207–212. PubMed PMID: 1867954.
19. Barltrop J, Owen T. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. Bioorg Med Chem Lett. 1991;1:611–614.
20. Paull KD, Shoemaker RH, Boyd MR, et al. The synthesis of XTT: A new tetrazolium reagent that is bioreducible to a water-soluble formazan. J Heterocyclic Chem. 1988;25:911–914.
21. Ishiyama M, Shiga M, Sasamoto K, Mizoguchi M, He P. A new sulfonated tetrazolium salt that produces a highly water-soluble formazan dye. Chem Pharm Bull (Tokyo). 1993;41:1118–1122.

22. Tominaga H, Ishiyama M, Ohseto F, et al. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun. 1999;36:47–50.
23. Goodwin CJ, Holt SJ, Downes S, Marshall NJ. 1995. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. J Immunol Methods 13;179(1):95-103.
24. Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res. 1988;48(17):4827–33. PubMed PMID: 3409223.
25. Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnology Annual Review. 2005;11:127–152. PubMed PMID: 16216776.
26. Ahmed SA, Gogal RM, Walsh JE. A new rapid and simple nonradioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [3H]thymidine incorporation assays. J Immunol Meth. 1994;170:211–224. PubMed PMID: 8157999.
27. Shum D, Radu C, Kim E, Cajuste M, Shao Y, Seshan VE, Djaballah H. A high density assay format for the detection of novel cytotoxic agents in large chemical libraries. J Enz Inhib Med Chem. 2008;23(6):931–945. PubMed PMID: 18608772.
28. Wesierska-Gadek J, Gueorguieva M, Ranftler C, Zerza-Schnitzhofer G. A new multiplex assay allowing simultaneous detection of the inhibition of cell proliferation and induction of cell death. J Cell Biochem. 2005;96(1):1–7. PubMed PMID: 16052484.
29. Invitrogen alamarBlue Assay manual. Online at [Available at: http://tools.invitrogen.com/content/sfs/manuals/PI-DAL1025-1100_TI%20alamarBlue%20Rev%201.1.pdf].
30. Lancaster MV and Fields RD. Antibiotic and cytotoxic drug susceptibility assays using resazurin and poising agents. US Patent 5,501,959. Issued March 26, 1996.
31. Niles AL, Moravec RA, Hesselberth PE, Scurria MA, Daily WJ, Riss TL. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. Anal Biochem. 2007;366:197–206. PubMed PMID: 17512890.
32. CellTiter-Fluor® Technical Bulletin. Online at Available at: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/101/CellTiter-Fluor%20Cell%20Viability%20Assay%20Protocol.pdf
33. Caspase-Glo® Technical Bulletin. Online at Available at: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/101/Caspase-Glo%203%207%20Assay%20Protocol.pdf
34. PubChem BioAssay.: http://pubchem.ncbi.nlm.nih.gov/assay/
35. Hall MP, Gruber MG, Hannah RR, Jennens-Clough ML, Wood KV. 1998. Stabilization of firefly luciferase using directed evolution. In: Bioluminescence and Chemiluminescence—Perspectives for the 21st Century. Roda A, Pazzagli M, Kricka LJ, Stanley PE (eds.), pp. 392–395. John Wiley & Sons, Chichester, UK.

36. Auld DS, Zhang Y-A, Southall NT, et al. A basis for reduced chemical library inhibition of firefly luciferase obtained from directed evolution. J Med Chem. 2009;52:1450–1458. PubMed PMID: 19215089.
37. CellTiter-Glo® Technical Bulletin #288. Online at Available at: http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Bulletins/101/Caspase-Glo%203%207%20Assay%20Protocol.pdf.
38. Riss TL, Moravec RA. Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. Assay Drug Dev Technol. 2004;2(1):51–62. PubMed PMID: 15090210.
39. Duellman SJ, Zhou W, Meisenheimer P, Vidugiris G, Cali JJ, Gautam P, Wennerberg K, Vidugiriene J. Bioluminescent, Nonlytic, Real-Time Cell Viability Assay and Use in Inhibitor Screening. Assay Drug Dev Tech. 2015;13(8):456–65. PubMed PMID: 26383544.
40. RealTime-Glo MT Cell Viability Assay Technical Manual. Online at Available at: http://www.promega.com/~/media/files/resources/protocols/technical%20manuals/101/realtimeglo%20mt%20cell%20viability%20assay%20protocol.pdf