

Introducing the CellTiter-Blue™ Cell Viability Assay

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Abstract

To assist researchers in measuring cell viability *in vitro*, Promega introduces the CellTiter-Blue™ Cell Viability Assay. The assay provides a homogeneous, fluorometric method for estimating the number of viable cells in multiwell plates. The simple procedure involves addition of a single reagent directly to cells cultured in serum-supplemented medium. This article describes the assay and highlights its advantages for automated cytotoxicity screening.

The CellTiter-Blue™ Cell Viability Assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). The homogeneous procedure involves addition of a single reagent directly to cells in culture.

Introduction

The viability of a population of cells *in vitro* can be determined using a variety of experimental methods. One parameter used to define cell viability is whether or not metabolic processes remain active. Viable cells must carry out metabolic reactions to generate the energy required to maintain homeostatic processes, including synthesis of critical components and maintenance of membrane potential. When cells lose membrane integrity *in vitro*, their ability to carry out metabolic processes ceases; in other words, they die. One method for monitoring cell viability is the conversion of indicator dyes to form a measurable end product.

The CellTiter-Blue™ Assay

The CellTiter-Blue™ Cell Viability Assay provides a homogeneous, fluorometric method for monitoring cell viability. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). The CellTiter-Blue™ Reagent is a buffered solution containing highly purified resazurin. The conditions for using resazurin reduction as an indicator of cell viability and the other ingredients of the CellTiter-Blue™ Reagent have been optimized for use as a cell viability assay. Viable cells retain the ability to reduce resazurin into resorufin (Figure 1). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal.

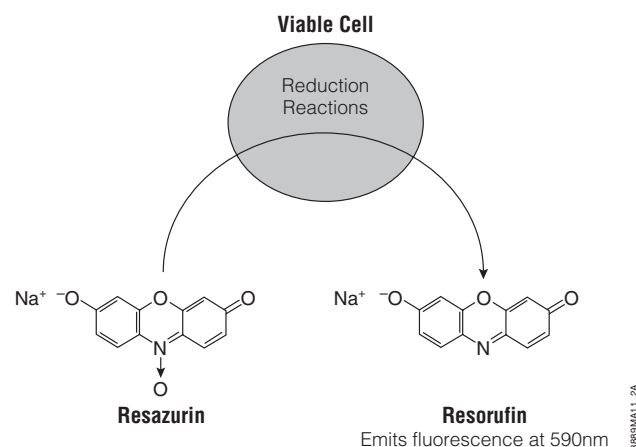


Figure 1. Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product. The Fluorescence produced is proportional to the number of viable cells.

The CellTiter-Blue™ Reagent was designed for use as an endpoint assay rather than as a kinetic method of monitoring cell growth. The Reagent should be added near the end of the period of exposure to the compound being tested.

The spectral properties of the CellTiter-Blue™ Reagent change upon reduction of resazurin to resorufin. Resazurin is dark blue and has little intrinsic fluorescence until it is reduced to resorufin, which is pink and highly fluorescent. The visible light absorbance spectrum of the CellTiter-Blue™ Reagent undergoes a “blue shift” upon reduction of resazurin to resorufin. The absorbance maximum of resazurin is 605nm, and the reduced resorufin has a maximum at 573nm. Either fluorescence or absorbance may be used to record assay results; however fluorescence is the preferred method because it is more sensitive.

A flow diagram summarizing the CellTiter-Blue™ Assay protocol is shown in Figure 2. The homogeneous assay procedure involves addition of a single reagent directly to cells cultured in serum-supplemented medium. After an incubation step, data are recorded using either a plate-reading fluorometer or spectrophotometer. Cell washing, removal of medium and multiple pipetting steps are not required, making the assay ideal for adaptation to automated high-throughput screening assays for cell viability and cytotoxicity.

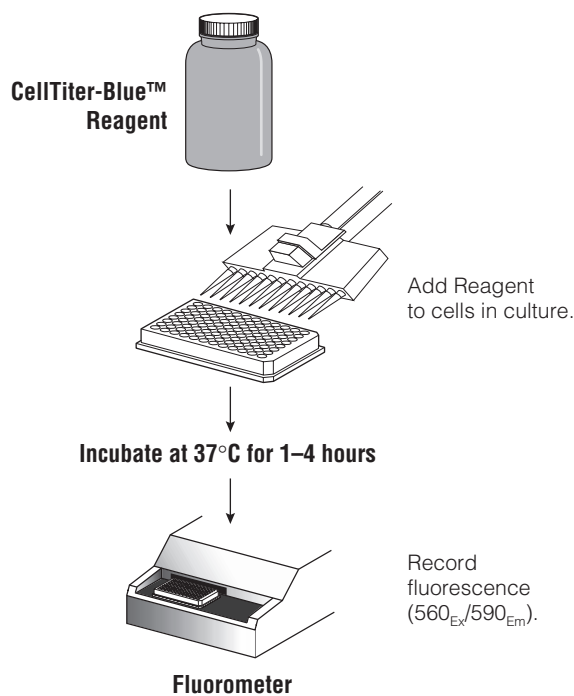


Figure 2. The CellTiter-Blue™ Cell Viability Assay protocol. Multiwell plates (96- or 384-well) compatible with fluorescent plate readers are prepared with cells and the compounds to be tested using standard methods. CellTiter-Blue™ Reagent is added directly to each well, the plates are incubated at 37°C to allow cells to convert resazurin to resorufin, and the fluorescent signal is measured.

Linear Range and Sensitivity

Under most experimental conditions, the fluorescent signal from the CellTiter-Blue™ Reagent is proportional to the number of viable cells (Figure 3). The linear range and lower limit of detection are dependent on the cell type. For example, HepG2 cells have a higher metabolic rate than Jurkat cells and thus a greater capacity to reduce resazurin.

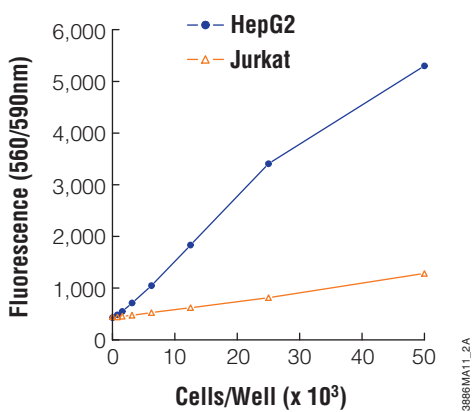


Figure 3. Relative ability of different cell types to reduce resazurin. Serial twofold dilutions of Jurkat or HepG2 cells were prepared at 100 μ l/well in a 96-well plate and cultured for 1.5 hours at 37°C. CellTiter-Blue™ Reagent (20 μ l/well) was added and cells were incubated for 1 hour before recording fluorescence 560(20)_{Ex}/590(10)_{Em} using a Labsystems Fluoroskan Ascent plate reader.

The ability of different cell types to reduce resazurin varies depending on the metabolic capacity of the cells and the length of incubation with the CellTiter-Blue™ Reagent. For most applications a 1- to 4-hour incubation period is adequate. To optimize performance for screening assays, the number of cells/well and the length of the incubation period should be empirically determined.

The lower limit of detection and the range of linear responsiveness are also dependent on incubation time. Figure 4 shows that extending the incubation period to 22 hours improves the signal-to-background ratio for lower cell numbers and increases assay sensitivity. However, longer incubation times may result in reduction of the dynamic range of the assay. The data in Figure 4 show that the lower limit of detection for a 4-hour incubation was 390 cells/well. When the incubation period was increased to 22 hours, the lower limit of detection improved (49 cells/well), but the relationship between fluorescence and cell number was not linear above 12,500 cells/well.

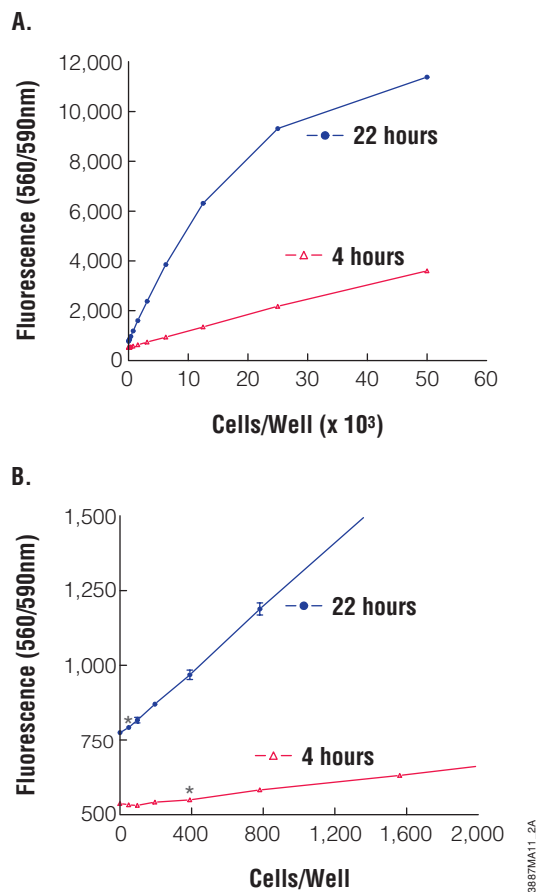


Figure 4. Effect of CellTiter-Blue™ Assay incubation time on signal formation. Serial twofold dilutions of Jurkat cells from 0–50,000 were incubated with CellTiter-Blue™ Reagent for 4 hours and 22 hours. **Panel A.** The 4-hour incubation period shows a linear correlation ($r^2 = 0.99$) between fluorescence and cell number. The lower limit of detection was 390 cells/well. For the 22-hour incubation period, there is a gain in assay sensitivity with a lower limit of detection of 49 cells/well, but a loss of linearity above 12,500 cells/well. **Panel B.** Detail for 0–2,000 cells per well. For the 4-hour incubation, the signal from 390 cells* was greater than that from zero cells + 3 standard deviations. For the 22-hour incubation, the signal from 49 cells* was greater than that from zero cells + 3 standard deviations ($n = 4$).

Simplifying Cytotoxicity Screening... continued

Comparison with Other Assay Methods

There is an excellent correlation between the CellTiter-Blue™ Assay and other methods for determining cell viability or cytotoxicity. We have compared results obtained using the CellTiter-Blue™ Assay with those obtained using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay^(a) (Cat.# G3582), which uses the reduction of MTS tetrazolium to a colored formazan product to measure cell viability, and the CellTiter-Glo™ Luminescent Cell Viability Assay^(b) (Cat.# G7570), which measures ATP content. There was excellent correlation between IC₅₀ values obtained with all three assay methods (1).

Figure 5 shows the effect of tamoxifen on HepG2 cells. These data were generated using the CellTiter-Blue™ Assay and the CytoTox-ONE™ Homogeneous Membrane Integrity Assay^(b) (Cat.# G7890), which measures release of lactate dehydrogenase (LDH), a marker of membrane integrity commonly used in cytotoxicity assays. Increasing concentrations of tamoxifen are toxic to HepG2 cells, resulting in a decrease in fluorescent signal in the CellTiter-Blue™ Assay. There is an inverse correlation between resazurin reduction as a viability indicator and release of LDH as a cytotoxicity indicator. The IC₅₀ values determined using both assay methods were similar.

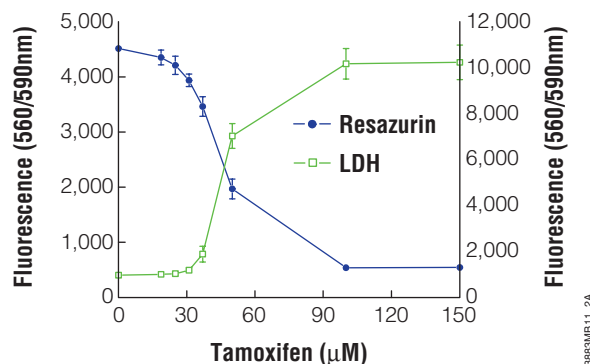


Figure 5. Comparison of CellTiter-Blue™ Reagent with the LDH-based CytoTox-ONE™ Homogeneous Membrane Integrity Assay. HepG2 cells were seeded at 15,000 cells/well in 96-well plates in 90µl MEM supplemented with 10% FBS, nonessential amino acids and 1mM sodium pyruvate and were cultured for 24 hours at 37°C. Tamoxifen (0–150µM) was diluted into culture medium. Plates designated for the CytoTox-ONE™ (LDH) Assay were cooled to 22°C prior to addition of the CytoTox-ONE™ Reagent. For CellTiter-Blue™ Assays, plates were incubated for 1 hour at 37°C after addition of the CellTiter-Blue™ Reagent.

Volume of CellTiter-Blue™ Reagent Used

The CellTiter-Blue™ Reagent has been designed to use convenient volumes for accurate pipetting in 96- and 384-well plates. Twenty microliters of CellTiter-Blue™ Reagent is added to each 100µl of medium in a 96-well format, or 5µl of Reagent is added to each 25µl of culture medium in a 384-well format. Using a Labsystems Fluoroskan Ascent plate reader with a 560(20)_{Ex}/590(10)_{Em} filter, this ratio of CellTiter-Blue™ Reagent to culture medium resulted in greater fluorescent signal and reduced fluorescent background compared to other commercially available resazurin-based assays. The ratio of CellTiter-Blue™ Reagent to cell culture volume can be adjusted for optimum performance.

Site of Resazurin Reduction

Microscopy has shown that resazurin is reduced to resorufin inside living cells (2). Resazurin can penetrate cells, where it becomes reduced to the fluorescent resorufin, probably resulting from the action of several different redox enzymes. The fluorescent resorufin can diffuse into the surrounding medium. Culture medium harvested from rapidly growing cells does not reduce resazurin (2). An analysis of various hepatic subcellular fractions suggests that resazurin can be reduced by mitochondrial, cytosolic and microsomal enzymes (3).

Assay Controls

To check for intrinsic fluorescence of test compounds or chemical interference with resazurin reduction, we recommend setting up negative control wells containing culture medium without cells. Add the same solvent used to deliver the test compounds to the negative control wells to test for possible interference with assay chemistry. As a positive control for cytotoxicity assays, we recommend including a set of wells containing cells treated with a compound known to be toxic to the cells used in your model system.

Optical Properties of Resazurin and Resorufin

Both the light absorbance and fluorescence properties of the CellTiter-Blue™ Reagent are changed by cellular reduction of resazurin to resorufin, thus either absorbance or fluorescence measurements can be used to monitor results. The absorption maximum for resazurin in RPMI 1640 + 10% fetal bovine serum is 605nm, and the absorption maximum for resorufin is 573nm (1). If absorbance measurements are used to record data, we recommend taking readings at 570nm and using 600nm as a reference wavelength. Values can be compared to blank wells containing CellTiter-Blue™ Reagent without cells. The absorption maxima for resazurin and resorufin are relatively broad, and alternative wavelengths may prove useful if your instrument does not contain the 570nm and 600nm filters.

We recommend fluorescence for recording data because it is more sensitive and allows a greater dynamic range. Options for fluorescence filter sets include 530–570nm for excitation and 580–620nm for emission.

Multiplexing with Other Assays

Because the CellTiter-Blue™ Reagent is relatively non-destructive to cells during short-term exposure, it is possible to use the same culture wells to do more than one type of assay. An example showing the measurement of cell viability using the CellTiter-Blue™ Reagent and measurement of apoptosis using the Apo-ONE® Homogeneous Caspase-3/7 Assay^(c) (Cat.# G7790) is shown in Figure 6.

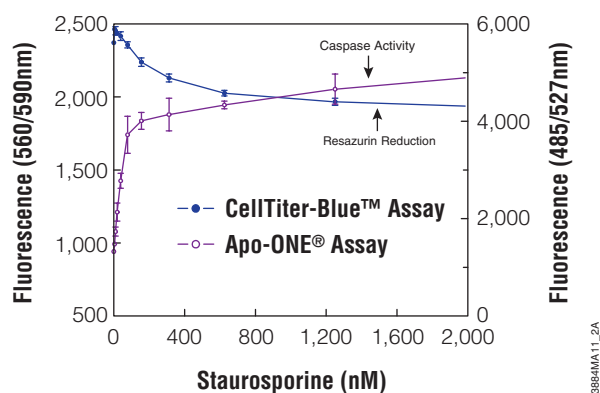


Figure 6. Multiplexing two assays in the same well. Jurkat cells (100µl/well) were treated with various concentrations of staurosporine for 5 hours. CellTiter-Blue™ Reagent (20µl) was added to each well immediately after drug addition and incubated simultaneously for 5 hours prior to recording fluorescence (560_{Ex}/590_{Em}). Then caspase activity was measured in the same wells by adding 120µl of the Apo-ONE® Homogeneous Caspase 3/7 Assay Reagent. Cells were incubated for an additional 1 hour at ambient temperature prior to recording fluorescence (485_{Ex}/527_{Em}).

Background Fluorescence and Light Sensitivity of Resazurin

The resazurin dye in the CellTiter-Blue™ Reagent and the resorufin product are light-sensitive. Prolonged exposure of the CellTiter-Blue™ Reagent to light will result in increased background fluorescence and decreased sensitivity. Background fluorescence can be corrected by including control wells on each plate to measure the fluorescence from serum-supplemented culture medium in the absence of cells. There may be a slight increase in background fluorescence in wells without cells after several hours of incubation. For additional technical information, please see the CellTiter-Blue™ Cell Viability Assay Technical Bulletin (1).

Summary

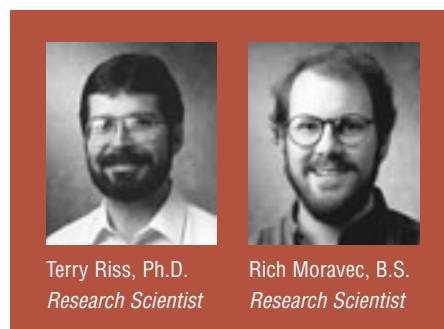
The homogeneous format and fluorescent detection method of the CellTiter-Blue™ Cell Viability Assay are ideally suited for automated screening. The CellTiter-Blue™ Assay was developed under Promega's ISO 9001 quality standards. The reagent is manufactured using traceable raw materials and strict quality controls that ensure the high purity necessary to earn the CellTiter name. This product represents another significant addition to Promega's line of homogeneous cell viability, cytotoxicity and apoptosis detection assays.

References

1. CellTiter-Blue™ Cell Viability Assay Technical Bulletin #TB317, Promega Corporation.
2. O'Brien, J. *et al.* (2000) *Eur. J. Biochem.* **267**, 5421–6.
3. Gonzalez, R.J. and Tarloff, J.B. (2001) *Toxicology in Vitro* **15**, 257–9.

Protocol

- ◆ CellTiter-Blue™ Cell Viability Assay Technical Bulletin #TB317, Promega Corporation. (www.promega.com/tbs/tb317/tb317.html)



Ordering Information

Product	Size	Cat.#
CellTiter-Blue™ Cell Viability Assay	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082

^(a) The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.

^(b) Patent Pending.

^(c) This product is covered by U.S. Pat. Nos. 4,557,862 and 4,640,893 and is sold for research use only. All other uses, including but not limited to use as a clinical diagnostic or therapeutic, require a separate license. Please contact Promega Corporation for details relating to obtaining a license for such other use.

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