

A Viable Solution for Cytotoxicity Screening



CellTiter-Glo™ Luminescent Cell Viability Assay: Fast, Sensitive and Flexible

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Abstract

Promega's CellTiter-Glo™ Luminescent Cell Viability Assay is a highly sensitive method for assaying cell proliferation and cytotoxicity. The assay uses a unique, stable form of luciferase to measure ATP as an indicator of viable cells. The luminescent signal produced is proportional to the number of viable cells present in culture. The homogeneous format of the assay is well suited for high-throughput applications and is scalable from 96-, 384- and 1536-well formats with little or no loss in sensitivity. Results can be achieved rapidly, while the extended glow luminescence feature offers flexibility in processing large batches of plates.

A single addition of CellTiter-Glo™ Reagent directly to cells in culture eliminates the need for multiple pipetting or cell washing steps.

How the Assay Works

The CellTiter-Glo™ Luminescent Cell Viability Assay^(a) uses luciferase to measure ATP as an indicator of metabolically active, "viable" cells (1–3). The assay is designed to measure the number of viable cells in standard laboratory or high-throughput applications, including cell proliferation and cytotoxicity assays and the increasingly popular applications for ADME/Tox screening (Absorption, Distribution, Metabolism, Elimination and Toxicity; 1).

The CellTiter-Glo™ Reagent is prepared by reconstituting the lyophilized CellTiter-Glo™ Substrate with the CellTiter-Glo™ Buffer. The final volume of CellTiter-Glo™ Reagent used depends upon the final volume of cells and test compounds. A volume of CellTiter-Glo™ Reagent equal to the volume of cells is added to the culture wells. The plates are agitated briefly and the luminescence detected with a plate-reading luminometer or CCD camera. Figure 1 shows a flow diagram depicting the CellTiter-Glo™ Assay protocol. A single addition of CellTiter-Glo™ Reagent directly to cells in culture eliminates the need for multiple pipetting or cell washing steps.

After adding the CellTiter-Glo™ Reagent and mixing, results are obtained in just 10 minutes. This is advantageous over several other cell viability assays that require cells to metabolize substrates such as calcein-AM or tetrazolium reagents to generate a signal. The CellTiter-Glo™ Assay eliminates the need for extended incubations and can give a better indication of cell viability following

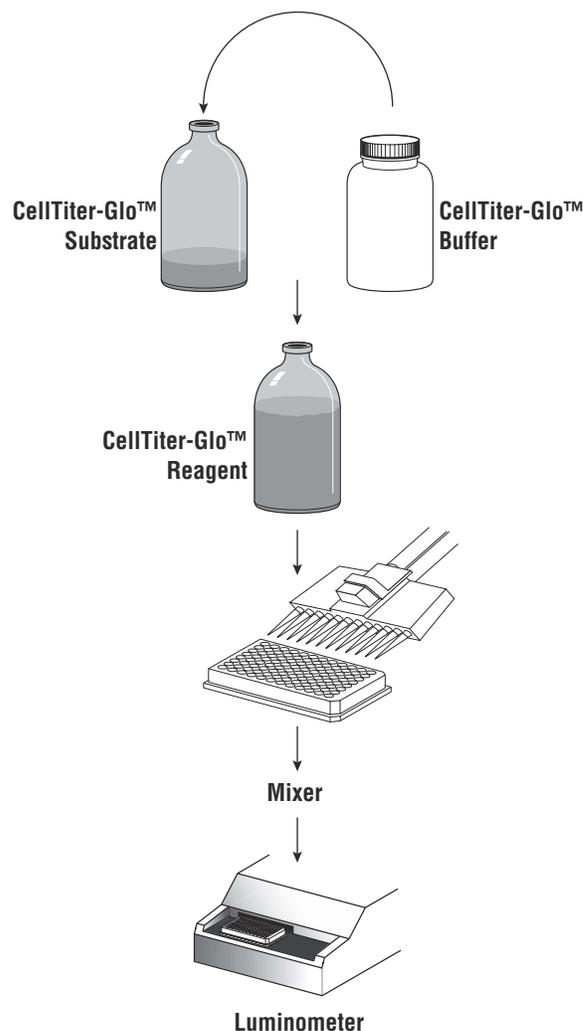


Figure 1. Flow chart depicting the CellTiter-Glo™ Luminescent Cell Viability Assay protocol.

drug treatments, particularly in situations where cells are treated for short periods of time.

The luciferase present in the CellTiter-Glo™ Reagent uses luciferin, oxygen and ATP as substrates in a reaction that produces oxyluciferin and releases energy in the form of light. Because the luciferase reaction requires ATP, conditions have been created such that the amount of light produced is proportional to the amount of ATP present, reflecting the number of viable cells. As shown in Figure 2, luminescence correlates directly with cell number. The data show that the CellTiter-Glo™ Assay is able to detect 4 Jurkat cells/well ($p < 0.001$) with an r^2 value of 0.99 up to 4,000 cells/well in a 384-well format.

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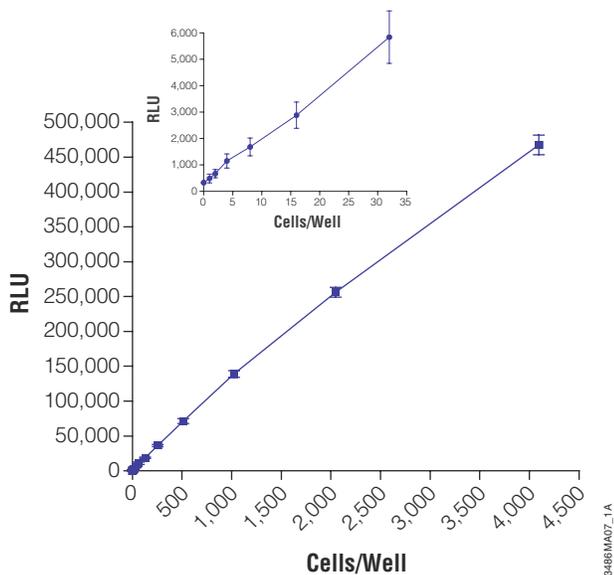


Figure 2. Cell number correlates with luminescent output. There is a direct relationship between luminescence and cell number as measured with the CellTiter-Glo™ Assay. Serial two-fold dilutions of Jurkat cells in RPMI + 10% FBS were made in a 384-well opaque white plate as 25µl/well samples. An equal volume of CellTiter-Glo™ Reagent was added and mixed, and the luminescence was recorded at 10 minutes using a Wallac Victor™ 1420 multilabel counter. Values represent the mean and S.D. of 8 replicates of each cell number ($r^2 = 0.99$ between 0–4,000 cells/well). A Student's t-test indicated that luminescence from 4 cells is significantly above background ($p < 0.001$). RLU = Relative Light Units.

The linear range is extended up to 50,000 cells/well in a 96-well format (not shown). The limit of sensitivity and linear range with other cells may vary.

ATP measurement is becoming more widely accepted as an indicator of the number of viable cells present. A recent report from the Multicenter Evaluation of in vitro Cytotoxicity (MEIC) found ATP concentration assays to be among the most predictive general toxicity methods (2). When cell viability is lost and membrane integrity is compromised, there is a rapid drop in the level of ATP present, resulting from the combination of a loss of the cell's ability to synthesize more ATP and removal of any remaining ATP by the action of ATPases. Figure 3 illustrates the rapid loss of ATP from a population of cells that have been treated to compromise the cell membrane. These data further support the validity of using ATP as a marker of the presence of viable cells.

The CellTiter-Glo™ Assay uses a thermostable form of luciferase that is proprietary to Promega. This unique luciferase enables assay conditions that inhibit endogenous ATPase activity while allowing the luciferase reaction to proceed for several hours. The stability of this unique form of luciferase allows the researcher to perform the assay with a single reagent addition step using a homogeneous format. The CellTiter-Glo™ Reagent inhibits endogenous ATPases and provides the substrates and conditions necessary to sustain light output for several hours. Figure 4 illustrates

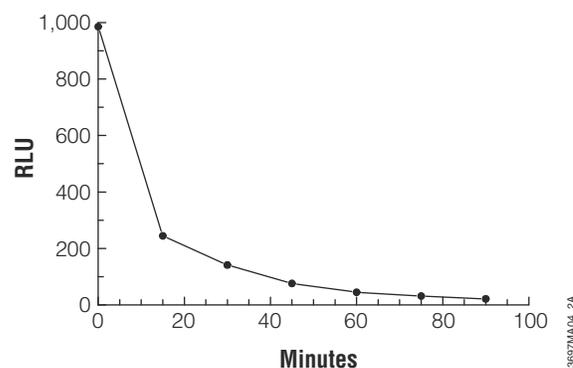


Figure 3. ATP levels drop rapidly upon cell lysis under normal culture conditions. CHO-K1 cells (15,000/well) were plated into a 96-well plate in DME/F-12 + 10% FBS and allowed to attach overnight at 37°C, 5% CO₂. Wells (n = 4) were treated with staggered additions of 0.2% saponin to lyse cells and incubated at 37°C for the indicated times to allow ATPase activity to occur. CellTiter-Glo™ Reagent was then added to measure ATP in all samples simultaneously. RLU = Relative Light Units.

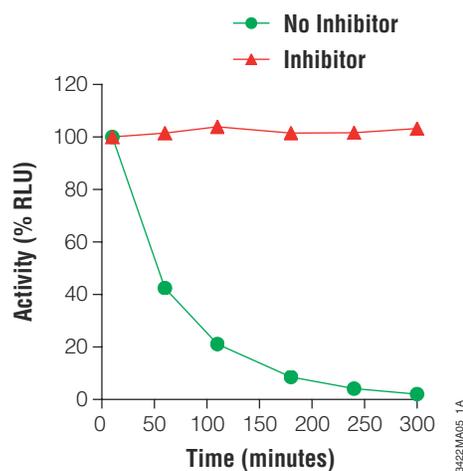


Figure 4. Inhibition of ATPase activity by the CellTiter-Glo™ Reagent. Cell lysates were prepared by 3–4 freeze-thaw cycles of 1.5×10^6 L929 cells/ml in DME/F-12 (1:1) containing 10% horse serum. Two pools of cell lysate were incubated at 22°C. To one pool, we added an equal volume of 50mM HEPES (pH 7.5; no inhibitor), and to the other we added an equal volume of CellTiter-Glo™ Buffer (inhibitor). At five 60-minute intervals, 100µl aliquots were removed and 20µl 5X CellTiter-Glo™ Substrate was added and mixed. Luminescent output was measured. Quadruplicate samples were taken for each time point.

the protection from ATPases conferred by the presence of an inhibitor in the CellTiter-Glo™ Buffer. The signal generated from the CellTiter-Glo™ Assay results from a “glow” type luminescent reaction with an extended half-life typically greater than 5 hours, depending on cell type, medium and serum used (Figure 5). This long half-life eliminates the need for luminometers with reagent injectors and allows flexibility in plate processing.

Comparison with Accepted Methods for Cytotoxicity Assays

The measurement of ATP correlates well with other accepted viability assay methods. Figure 6 shows a TNFα cytotoxicity assay and compares the detection methods

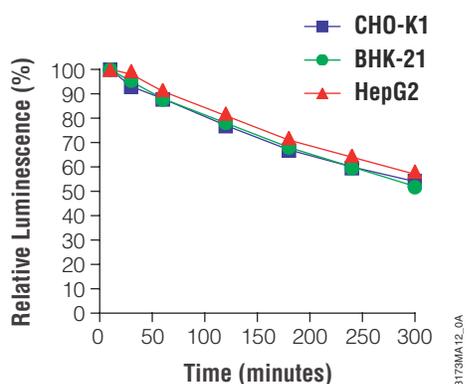


Figure 5. The half-life of luminescent signal produced by CellTiter-Glo™ Reagent is greater than 5 hours for the cell lines shown. The long half-life of luminescent signal provides the flexibility of performing “continuous” or “batch mode” handling of assay plates for high-throughput screening (HTS).

of the CellTiter-Glo™ Assay and the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay^(b), a colorimetric assay that measures MTS tetrazolium reduction. Although each assay measures a different indicator of cellular metabolism, both assays give a similar ED₅₀ value (~8pg/ml). We have tested the CellTiter-Glo™ Assay with a variety of cells including Jurkat, HepG2, BHK-21, CHO-K1 and SH-SY5Y cells. Although there are differences in the absolute amounts of ATP depending on cell type, all cell types tested so far have worked well with this assay.

The flexibility of the CellTiter-Glo™ Assay is illustrated in Figure 6, Panel A, which shows a CCD camera image obtained from the same TNFα cytotoxicity assay plate that was used for the luminometer readings plotted in Panel B. A slightly modified fluorometer also has been used successfully to collect luminescent data from the CellTiter-Glo™ Assay. The light from the excitation lamp was blocked using a solid opaque filter, and the emission filter was removed to allow the maximum amount of light to pass through to the photomultiplier tube.

Scalable from Bench to HTS formats

With the increased availability of equipment designed to accommodate 384-well plates, many laboratories have scaled their screening methods to this smaller format. Because of this miniaturization, cell viability assays need to be sensitive, fast and adaptable. The CellTiter-Glo™ Luminescent Cell Viability Assay meets all of these criteria.

The CellTiter-Glo™ Assay is scalable from 96- and 384- to 1536-well formats, making it convenient for miniaturization and high-throughput screening. The culture volume has little effect on assay results. Statistical analysis of data from a 1536-well plate assay showing the Z' factor of 0.84 demonstrates the power of the CellTiter-Glo™ Assay to distinguish between negative and positive control samples (Figure 7).

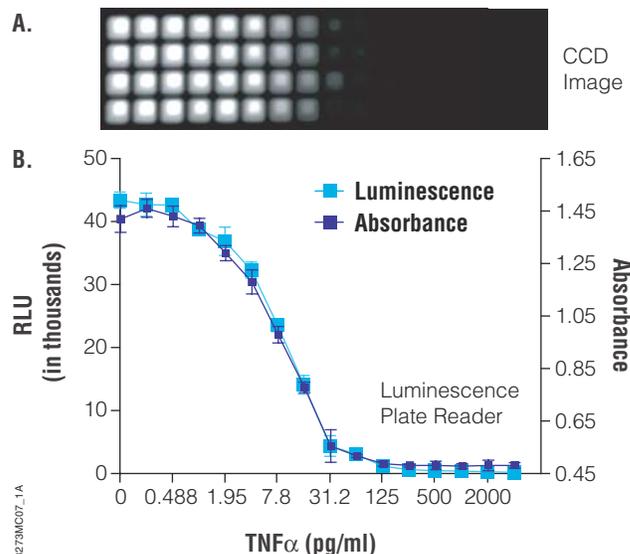


Figure 6. TNFα cytotoxicity of L929 cells is similar when measured with the CellTiter-Glo™ Assay or the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay in 384-well plates. L929 cells (1,000 cells/well) were plated in a solid white and a clear-bottom 384-well plate and allowed to attach and grow for 24 hours. Varying concentrations of TNFα (n = 4) were added in the presence of actinomycin D (1µg/ml final concentration) and incubated for 20 hours. Cytotoxicity was determined by adding an equal volume (30µl/well) of CellTiter-Glo™ Reagent, shaking the plate and recording the luminescence 10 minutes later using a Berthold® Orion® plate luminometer. Alternatively, 6µl per well of CellTiter 96® AQ_{ueous} One Solution Reagent was added to wells of the clear-bottom plate. This plate was incubated for 2 hours at 37°C prior to determining absorbance at 490nm on a Wallac Victor™ 1420 multilabel counter. **Panel A:** Luminescence visualized with an Alpha Innotech Multi-Image Light Cabinet CCD camera (30-second exposure at medium sensitivity). Each column of replicates corresponds to the mean data point on the graph as determined by the luminometer. **Panel B:** The ED₅₀ values determined by both assays were approximately equivalent (~8pg/ml).

Reagent Stability and Interferences

The components of the CellTiter-Glo™ Assay are stable for 6 months if handled and stored properly. The reconstituted CellTiter-Glo™ Reagent is stable for several months if stored at -20°C and withstands the effects of multiple freeze-thaw cycles (3). There is minimal (~5%) loss of activity when the reconstituted reagent is stored at 4°C for 48 hours and a 20% loss of activity if the reconstituted Reagent is stored at 22°C for up to 48 hours.

The CellTiter-Glo™ Assay is robust and shows minimal or no detrimental effects on assay performance from phenol red or solvent vehicles commonly used to dissolve and deliver chemical compounds to assay plates. Common drug solvents such as DMSO, acetonitrile and ethanol have been shown to have little effect on assay performance.

Serum supplements of cell culture medium may affect luciferase assays. Slight differences in luminescence intensity of the CellTiter-Glo™ Assay have been observed using different types of culture medium and serum. Consistency within an assay should be achieved as long as the same culture medium formulation and serum supplement is used.

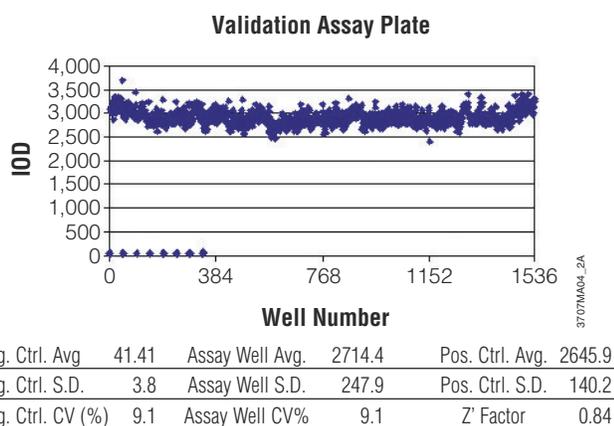


Figure 7. Scattergram of 1536-well validation assay plate using CellTiter-Glo™ Assay. A BlueBiRD automated reagent dispenser was used to dispense 1µl/well containing one thousand 293-EBNA cells into wells of a Corning 1536-well test plate. After a 24-hour incubation, 1µl CellTiter-Glo™ Reagent was added, and luminescence recorded using a ViewLux™ CCD camera. Integrated Optical Density units (IOD) are plotted along the y axis. The signal was consistent across the entire plate. The CV, Z' factor, and signal to background were within acceptable ranges for screening. Data provided by Karen Wilson and Robert Swanson, Pharmacopeia, Inc.

Effect of Temperature on Luminescent Signal

The intensity of the luminescent signal from the CellTiter-Glo™ Assay depends on the rate of the luciferase reaction. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, we recommend equilibrating assay plates to a constant temperature (22°C) prior to recording the luminescent signal. Transferring eukaryotic cells from 37°C to ambient temperature has little effect on the ATP content. We have demonstrated that removing cultured cells from a 37°C incubator and allowing them to equilibrate to 22°C for up to an hour prior to addition of CellTiter-Glo™ Reagent had little effect on the ATP content.

Plate Types

We recommend using standard opaque-walled 96- and 384-well plates suitable for luminescence measurements with the CellTiter Glo™ Assay. Opaque-walled plates with clear bottoms allowing microscopic visualization of cells may also be used; however, signal intensity may diminish slightly, and greater “cross talk” between wells has been observed.

Summary

The CellTiter-Glo™ Assay possesses many qualities that make it a flexible and robust assay, ideal for a variety of situations from the research laboratory to HTS drug discovery environments. The assay has several major advantages over other cell viability assays. It is a homogeneous method with a single reagent addition step. It is faster than other assays that require 1–4 hours of incubation, and it is sensitive enough to detect as few as 4 mammalian cells per well in a 384-well format. The

long half-life of the glow signal enables batch or continuous processing of multiple plates with little difference in light intensity among the plates, and the results correlate with other widely accepted methods of measuring cell viability.

References

1. Sussman, N. *et al.* (2002) *Drug Disc. Dev.* **5**, 71–72.
2. Ekwall, B. *et al.* (2000) *ATLA* **28**, Suppl. 1, 201–34.
3. Moravec, R. *et al.* (2001) *Cell Notes* **2**, 13–16.

Protocol

- ◆ *CellTiter-Glo™ Luminescent Cell Viability Assay Technical Bulletin* #TB295, Promega Corporation.
www.promega.com/tbs/tb288/tb288.html

Ordering Information

Product	Size	Cat.#
CellTiter-Glo™ Luminescent Cell Viability Assay ^(a)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter 96® AQ _µ eous One Solution Cell Proliferation Assay ^{*(b)}	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581

*For Laboratory Use.

^(a) Patent Pending.

^(b) 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.

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