TECHNICAL MANUAL

CellTiter-Glo® 2.0 Assay

Instructions for Use of Products
G9241, G9242 and G9243

Revised 1/23
TM403

CellTiter-Glo® 2.0 Assay

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The CellTiter-Glo® 2.0 Assay[a,b] provides a homogeneous method to determine the number of viable cells in culture by quantitating the amount of ATP present, which indicates the presence of metabolically active cells. This ready-to-use reagent is based on the original CellTiter-Glo® Luminescent Cell Viability Assay chemistry and eliminates the need to combine buffer with lyophilized substrate when preparing reagent. Although similar to the CellTiter-Glo® One Solution Assay, which needs to be stored frozen, the CellTiter-Glo® 2.0 Assay is much more stable and will maintain >85% light output upon storage at 4°C for 2 months or >85% light output upon storage at room temperature for 7 days (Figure 1). Functional performance (linearity, sensitivity and signal half-life) of the product is maintained at these temperatures for longer periods, although light output will decline over time. The CellTiter-Glo® 2.0 Assay is designed for use with multiwell-plate formats, making it ideal for automated high-throughput screening (HTS) and cell proliferation and cytotoxicity assays. The homogeneous assay procedure (Figure 2) involves addition of a single reagent (CellTiter-Glo® 2.0 Reagent) directly to cells cultured in serum-supplemented medium. Cell washing, removal of medium and multiple pipetting steps are not required.

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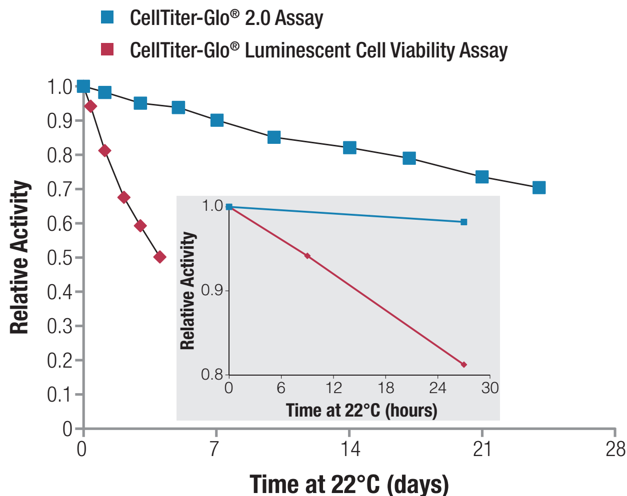
1. Description (continued)

A.



B.

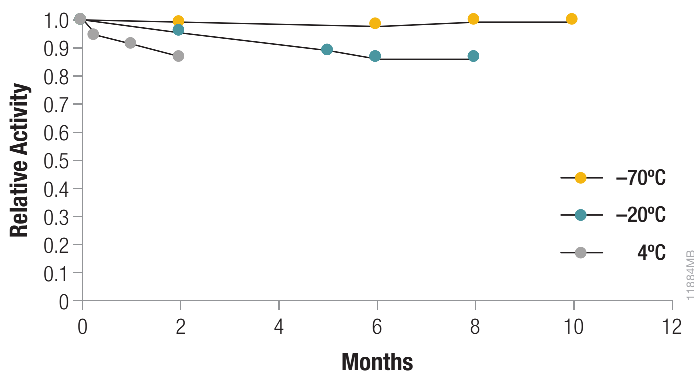


Figure 1. Reagent stability over time after storage at room temperature, 4°C, –20°C and –70°C. Panel A. Comparison of CellTiter-Glo® and CellTiter-Glo® 2.0 Reagents at 22°C. Aliquots of both reconstituted CellTiter-Glo® Reagent and CellTiter-Glo® 2.0 Reagent were incubated in a 22°C water bath for different lengths of time and then frozen at –80°C. After thawing, the reagent samples were mixed with an equal volume of 2µM ATP in water and luminescence was recorded at 10 minutes. The inset shows a magnification of the same data over a 24-hour period. The relative activity is the luminescence reported relative to the luminescence of the t = 0 sample. **Panel B.** CellTiter-Glo® 2.0 Reagent samples were stored at –70°C, –20°C or 4°C for different lengths of time. After equilibration to room temperature, the reagent samples were mixed with an equal volume of 1µM ATP in water, and luminescence was recorded at 10 minutes. The relative activity is the luminescence reported relative to the luminescence of the t=0 sample.

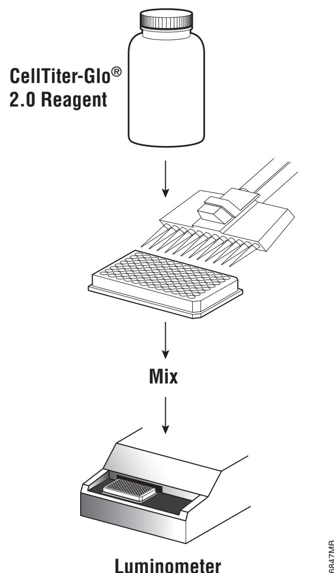


Figure 2. Overview of the CellTiter-Glo® 2.0 Assay protocol.

The luciferase reaction for this assay is shown in Figure 3. The "add-mix-measure" format results in cell lysis and generation of a luminescent signal that is proportional to the amount of ATP present (Figure 4). The amount of ATP is directly proportional to the number of cells present in culture (1). The CellTiter-Glo® 2.0 Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable "glow-type" luminescent signal and improves performance across a wide range of assay conditions. The half-life of the resulting luminescent signal is greater than 3 hours. This extended half-life eliminates the need for reagent injectors and provides flexibility for continuous or batch-mode processing of multiple plates.

The single-reagent-addition format reduces pipetting errors that may be introduced during the multiple steps required by other ATP-measurement methods.

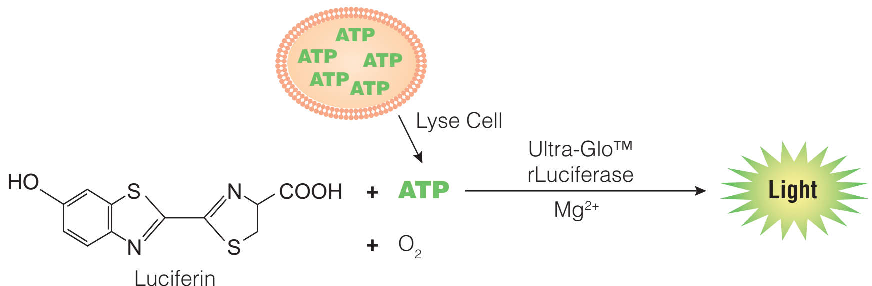


Figure 3. Overview of CellTiter-Glo® 2.0 Assay principle. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg^{2+}, ATP, which is contributed by viable cells, and molecular oxygen.

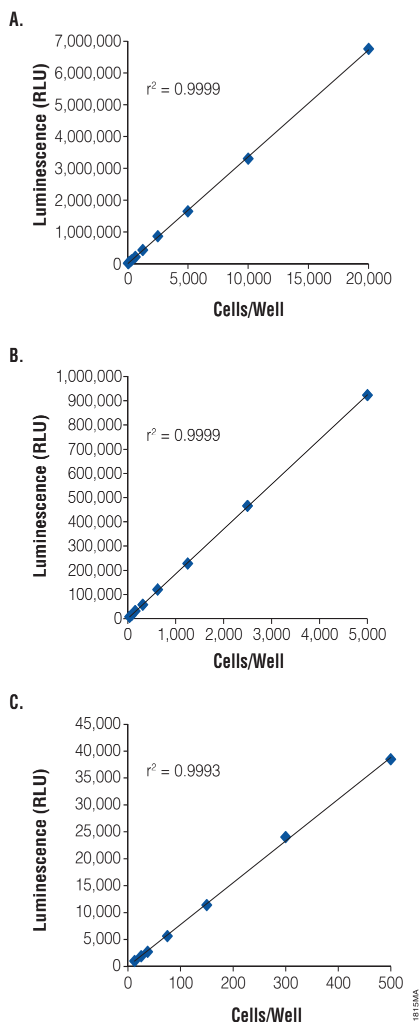


Figure 4. Cell number correlates with luminescent output. A direct relationship exists between luminescence measured with the CellTiter-Glo® 2.0 Assay and the number of cells in culture over three orders of magnitude. Serial twofold dilutions of Jurkat cells were made in RPMI 1640 with 10% FBS and plated in three formats. Volumes plated were 100µl of cells per well in a 96-well plate (**Panel A**), 25µl of cells per well in 384-well plate (**Panel B**) and 4µl of cells per well in a 1536-well plate (**Panel C**). An equivalent volume of CellTiter-Glo® 2.0 Reagent was dispensed into each well. The cells and reagent in the 1536-well plate were dispensed with automated equipment. Luminescence was recorded 10 minutes after addition of CellTiter-Glo® 2.0 Reagent. There is a linear relationship ($r^2 > 0.99$) between luminescent signal and cell number in each plate format.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CellTiter-Glo® 2.0 Assay	**10ml**	**G9241**

For in vitro Research Use Only. CellTiter-Glo® 2.0 Reagent is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates.

PRODUCT	SIZE	CAT.#
CellTiter-Glo® 2.0 Assay	**100ml**	**G9242**

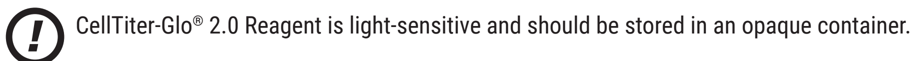
For in vitro Research Use Only. CellTiter-Glo® 2.0 Reagent is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates.

PRODUCT	SIZE	CAT.#
CellTiter-Glo® 2.0 Assay	**500ml**	**G9243**

For in vitro Research Use Only. CellTiter-Glo® 2.0 Reagent is sufficient for 5,000 assays at 100µl/assay in 96-well plates or 20,000 assays at 25µl/assay in 384-well plates.

Storage Conditions: Store at –30°C to –10°C. For maximum light signal, we recommend long-term storage at less than –65°C. Functional performance (linearity, sensitivity and signal half-life) of the reagent is maintained upon storage at –30°C to –10°C through the expiration date, although light output will decline over time.

CellTiter-Glo® 2.0 Reagent can be thawed and stored at +2°C to +10°C for up to 2 months with >85% light output remaining when measuring 1µM ATP in a standard reaction. Functional performance of the product is stable at these temperatures for longer, although light output will decline over time. The reagent can withstand four additional freeze-thaw cycles after the first thaw with no loss of light output. Do not refreeze the thawed reagent after extended storage above the label storage temperature (less than –10°C). We do not recommend dispensing CellTiter-Glo® 2.0 Reagent into aliquots due to the risk of ATP contamination.



CellTiter-Glo® 2.0 Reagent is light-sensitive and should be stored in an opaque container.

3. Performing the CellTiter-Glo® 2.0 Assay

Materials to Be Supplied by the User

- 22°C water bath
- opaque-walled multiwell plates adequate for cell culture
- multichannel pipette or automated pipetting station
- device (plate shaker) for mixing multiwell plates
- luminometer, CCD camera or imaging device capable of reading luminescence in multiwell plates
- **optional:** ATP for use in generating a standard curve in Section 3.C (Cat.# P1132, Sigma Cat.# A7699 or GE Healthcare Cat.# 27-1006)

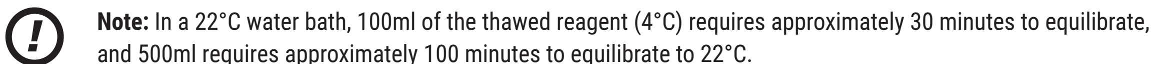
3.A. Reagent Preparation

1. If frozen, thaw CellTiter-Glo® 2.0 Reagent at 4°C overnight.

   Reagent may also be thawed in a 22°C water bath. Do not expose the reagent to temperatures above 25°C.

   If the CellTiter-Glo 2.0 Reagent is stored at –65°C, do not directly transfer it into a 22°C water bath to thaw. Instead, to avoid potentially cracking the bottle due to the rapid temperature change, leave the material on the bench top for 10–15 minutes and then place it in a 22°C water bath.

2. If not at room temperature, equilibrate the CellTiter-Glo® 2.0 Reagent to room temperature by placing the reagent in a 22°C water bath prior to use.



   Note: In a 22°C water bath, 100ml of the thawed reagent (4°C) requires approximately 30 minutes to equilibrate, and 500ml requires approximately 100 minutes to equilibrate to 22°C.

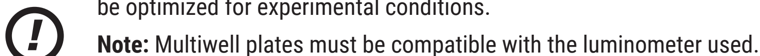
3. Mix gently by inverting the contents to obtain a homogeneous solution.

   Note: Use caution when removing the seal of the CellTiter-Glo® 2.0 Reagent bottle to avoid introducing ATP contamination.

3.B. Protocol for the Cell Viability Assay

Prepare and equilibrate the CellTiter-Glo® 2.0 Reagent as described in Section 3.A prior to performing the assay.

1. Prepare opaque-walled multiwell plates with mammalian cells in culture medium. Volumes and cell number should be optimized for experimental conditions.



   Note: Multiwell plates must be compatible with the luminometer used.

2. If desired, prepare control wells containing medium without cells to determine background luminescence.
3. Add test compound to experimental wells, and incubate according to your culture protocol.
4. Equilibrate the plate and its contents to room temperature for approximately 30 minutes.
5. Add a volume of CellTiter-Glo® 2.0 Reagent equal to the volume of cell culture medium present in each well (e.g., for a 96-well plate, add 100µl of CellTiter-Glo® 2.0 Reagent to 100µl of medium containing cells).
6. Mix the contents for 2 minutes on an orbital shaker to induce cell lysis (see Appendix for more information on mixing).
7. Allow the plate to incubate at room temperature for 10 minutes to stabilize the luminescent signal.
8. Record luminescence.

Notes:

a. Instrument settings depend on the manufacturer. Use an integration time of 0.25–1 second per well as a guideline.

b. Uneven luminescent signal within plates can be caused by temperature gradients, uneven seeding of cells or edge effects in multiwell plates.

3.C. Optional: Protocol for Generating an ATP Standard Curve

It is a good practice to generate a standard curve using the same plate on which samples are assayed. Because of endogenous ATPase enzymes found in serum, the ATP standard curve should be generated immediately before adding the CellTiter-Glo® 2.0 Reagent. If the amount of ATPase enzymes is sufficiently high, it may be necessary to omit serum from the medium used to generate the standard curve. We recommend ATP disodium salt (Cat.# P1132, Sigma Cat.# A7699 or GE Healthcare Cat.# 27-1006).

1. Prepare 1µM ATP in culture medium (100µl of 1µM ATP solution contains 10^{-10} moles of ATP).
2. Prepare serial tenfold dilutions of ATP in culture medium (1µM to 10nM; 100µl contains 10^{-10} to 10^{-12} moles of ATP, respectively).
3. Prepare a multiwell plate with varying concentrations of ATP standard in 100µl of medium (25µl for a 384-well plate).
4. Add a volume of CellTiter-Glo® 2.0 Reagent equal to the volume of ATP standard present in each well.
5. Mix contents for 2 minutes on an orbital shaker.
6. Allow the plate to incubate at room temperature for 10 minutes to stabilize the luminescent signal.
7. Record luminescence.

3.D. Sequential Multiplexing of CellTox™ Green Cytotoxicity Assay and CellTiter-Glo® 2.0 Assay

1. Completely thaw the CellTox™ Green Dye in a 37°C water bath. Mix the CellTox™ Green Dye using a vortex mixer to ensure homogeneity. A brief centrifugation may be necessary for complete recovery of the CellTox™ Green Dye. Although performance of CellTox™ Green Dye is optimal in black plates, white plates are optimal for sequential multiplexing formats that include a luminescent second measure.
2. Add the CellTox™ Green Dye at seeding or dosing so the final CellTox™ Green Dye concentration is 1X (stock = 1,000X) and incubate under desired conditions.
3. Equilibrate plate to room temperature. Measure fluorescence intensity at $485–500nm_{Ex}/520–530nm_{Em}$. Depending upon instrument manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker before measuring fluorescence.
4. Equilibrate the CellTiter-Glo® 2.0 Reagent to room temperature. After the final fluorescence measurement, add an equal volume of CellTiter-Glo® 2.0 Reagent to each well.
5. Place on an orbital shaker for 2 minutes and measure luminescence after 10 minutes.

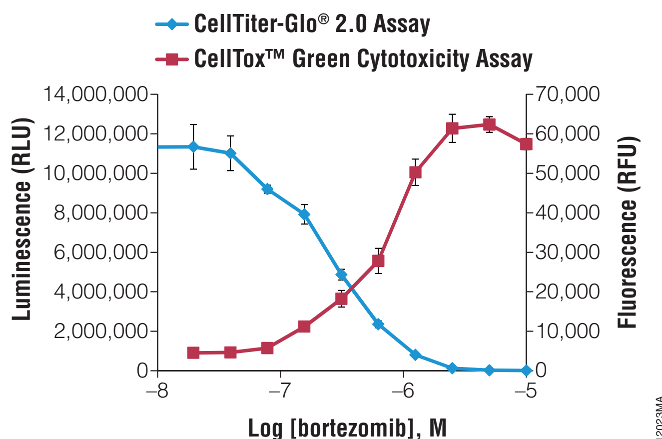


Figure 5. Multiplexing allows complementary measures of cell health. CellTox™ Green Reagent was applied to bortezomib-treated K562 cells after 48 hours of exposure. Fluorescence associated with cytotoxicity was measured, CellTiter-Glo® 2.0 Reagent was applied and luminescence associated with viability measured. These inverse measures produce similar EC_{50} values.

4. Appendix

4.A. Overview of the CellTiter-Glo® 2.0 Assay

The CellTiter-Glo® 2.0 Assay takes advantage of the properties of a proprietary thermostable luciferase, Ultra-Glo™ Recombinant Luciferase, to enable reaction conditions that generate a stable "glow-type" luminescent signal while simultaneously inhibiting endogenous enzymes released during cell lysis (e.g., ATPases). Release of ATPases will interfere with accurate ATP measurement. Historically, firefly luciferase purified from *Photinus pyralis* (LucPpy) has been used in reagents for ATP assays (1,4–7). However, LucPpy has only moderate stability in vitro and is sensitive to its chemical environment, including factors such as pH and detergents, limiting its usefulness for developing a robust homogeneous ATP assay. Promega has successfully developed a stable form of luciferase based on the gene from another firefly, *Photuris pennsylvanica* (LucPpe2) using an approach to select characteristics that improved performance in ATP assays. The unique characteristics of this mutant ($LucPpe2^m$), Ultra-Glo™ Recombinant Luciferase, enabled design of a homogeneous single-reagent-addition approach for performing ATP assays on cultured cells. Properties of CellTiter-Glo® 2.0 Assay overcome problems caused by factors such as ATPases that interfere with the measurement of ATP in cell extracts.

Sensitivity and Linearity: The ATP-based detection of cells is more sensitive than other methods (8–10). There is a linear relationship between luminescent signal and cell number in different plate formats. The luminescence values shown in Figures 1 and 4 were recorded after 10 minutes of incubation at room temperature to stabilize the luminescent signal as described in Section 3.B.

Incubation of sample plates for 3 hours at room temperature had little effect on the relationship between luminescent signal and cell number (data not shown). Moreover, this linearity is maintained upon storage. Figure 5 illustrates that even though the luminescent signal is slightly lower after storage for 4 months at 4°C relative to –20°C, there is an excellent linear relationship between luminescent signal and cell number at both storage temperatures.

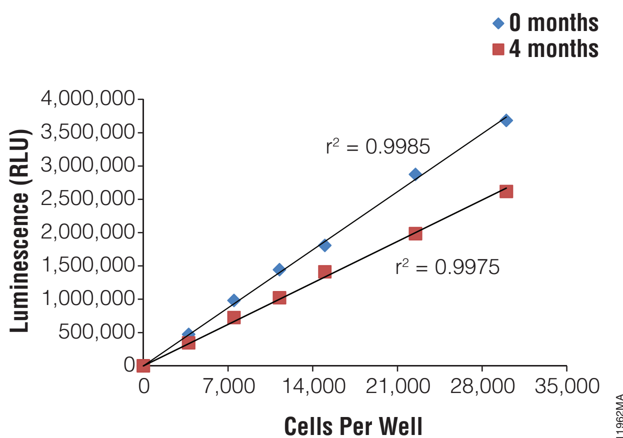


Figure 6. The correlation between cell number and luminescent signal is maintained upon CellTiter-Glo® Reagent storage. Aliquots of CellTiter-Glo® 2.0 Reagent were incubated at 4°C for 0 or 4 months (diamonds and squares, respectively) and then frozen at –80°C. After thawing, the reagent samples were mixed with a titration of Jurkat cells in RPMI 1640 with 10% FBS, and the luminescence was recorded at 10 minutes.

Automation Friendly: The Z-factor is a measure of assay precision and a metric commonly used to evaluate the suitability of an assay for screening applications (11). Any value greater than 0.5 is typically deemed acceptable, and the quality of the assay increases as the Z-factor approaches 1. To demonstrate the precision of the CellTiter-Glo 2.0® Assay, the reagent was used to assay an entire 384-well plate, with half the wells containing 600 Jurkat cells and the other half containing medium only (each well at a volume of 8µl). The Z-factor was calculated to be 0.81. A similar experiment was conducted with a 1536-well plate (half the wells contained 500 Jurkat cells and half contained medium only using volume of 4µl per well). For this experiment, the Z-factor was calculated to be 0.71. In both plate formats, the precision of the CellTiter-Glo 2.0® Assay is excellent.

Speed: The homogeneous procedure for measuring ATP using the CellTiter-Glo® 2.0 Assay is quicker than other ATP assay methods that require multiple steps for ATP extraction and measurement of luminescence. The CellTiter-Glo® 2.0 Assay also is faster than other commonly used methods for measuring the number of viable cells (such as MTT, alamarBlue® or Calcein-AM) that require prolonged incubation steps to enable the cellular metabolic machinery to convert indicator molecules into a detectable signal.

4.B. Additional Considerations

Temperature: The intensity and rate of decay of the luminescent signal from the CellTiter-Glo® 2.0 Assay depends on the luciferase reaction rate. Environmental factors that affect the luciferase reaction rate will change the intensity of light output and stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature before performing the assay. Transferring eukaryotic cells from 37°C to room temperature has little effect on ATP content (5). We have demonstrated that removing cultured cells from a 37°C incubator and allowing them to equilibrate to 22°C for 1–2 hours had little effect on ATP content. For batch-mode processing of multiple assay plates, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require a longer equilibration time than plates arranged in a single layer. Insufficient equilibration may result in a temperature gradient between wells in the center and on the edge of the plates. The temperature gradient pattern also may depend on the position of the plate in the stack.

Chemicals: The chemical environment of the luciferase reaction affects the enzymatic rate and thus luminescence intensity. Differences in luminescence intensity have been observed using different types of culture media and sera. The presence of phenol red in culture medium should have little effect on luminescence output. Solvents for the test compounds may interfere with the luciferase reaction and thus light output. To test for luciferase inhibition, assemble two reactions, one with equal volumes of CellTiter-Glo® 2.0 Reagent and 1µM ATP, and a second reaction with equal volumes of CellTiter-Glo® 2.0 Reagent and 1µM ATP plus the test compound. Incubate reactions for 10 minutes at 22°C–25°C, and then measure luminescence. A decrease in luminescence in the presence of test compound indicates luciferase inhibition.

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

Cellular ATP Content: Different cell types have different amounts of ATP, and values reported for the ATP level in a particular cell type vary considerably (1,4,12–14). Factors that affect the ATP content of cells may affect the relationship between cell number and luminescence. Anchorage-dependent cells that undergo contact inhibition at high densities may show a change in ATP content per cell at high densities, resulting in a nonlinear relationship between cell number and luminescence. Factors that affect cytoplasmic volume or cell physiology also will have an effect on ATP content. For example, oxygen depletion is one factor known to cause a rapid decrease in ATP (1).

Mixing: Optimum assay performance is achieved when the CellTiter-Glo® 2.0 Reagent is completely mixed with the cultured cells. Suspension cell lines (e.g., Jurkat cells) generally require less mixing to achieve lysis and extraction of ATP than adherent cells (e.g., L929 cells).

Several additional parameters related to reagent mixing include: the force of delivery of the CellTiter-Glo® 2.0 Reagent, sample volume and dimensions of the well. All of these factors may affect assay performance. The degree of reagent mixing required may be affected by the method used to add CellTiter-Glo® 2.0 Reagent to the assay plates. Automated pipetting devices that use a greater or lesser force of fluid delivery may affect the degree of subsequent mixing required. Complete reagent mixing in 96-well plates should be achieved using orbital plate shaking devices built into many luminometers and the recommended 2-minute shaking time. Special electromagnetic shaking devices using a radius smaller than the well diameter may be required to efficiently mix the contents of 384-well plates. The depth of medium and geometry of the multiwell plates may have an effect on mixing efficiency. We recommend that you consider these factors when performing the assay to determine whether a mixing step is necessary for your application.

ATP Contamination: Strict aseptic technique is essential to prevent ATP contamination of the CellTiter-Glo® 2.0 Reagent. Wear gloves and avoid contact with potentially contaminated surfaces and equipment. Clean gloves, lab surfaces and equipment with a 10% bleach solution, and then pat dry with lab wipes (e.g., Kimwipes® tissues). Use individually wrapped or designated ATP-free pipettes and pipette tips whenever possible, and avoid inserting pipettes or pipette tips into the CellTiter-Glo® 2.0 Reagent bottle multiple times. Discard any unused, dispensed reagent; do not return it to the original bottle.

Light Sensitivity: CellTiter-Glo® 2.0 is light sensitive and will decay more rapidly if exposed to light during storage. If the reagent is aliquotted or transferred from the original container, please be sure to protect the reagent from light.

4.C. References

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14. Andreotti, P.E. *et al.* (1995) Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay. *Cancer Res.* **55**, 5276–82.

4.D. Related Products

Viability Assays

Product	Size	Cat.#
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
CellTiter-Glo® 3D Assay	100ml	G9681
CellTiter-Glo® One Solution Assay	100ml	G8461
CellTiter-Glo® Luminescent Cell Viability Assay (luminescent)	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay (fluorescent)	10ml	G6080

Not for Medical Diagnostic Use. Additional kit formats are available.

Cytotoxicity Assays

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
	50ml	J2381
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CellTox™ Green Express Cytotoxicity Assay	200µl	G8731
CytoTox-Fluor™ Cytotoxicity Assay (fluorescent)	10ml	G9260
CytoTox-Glo™ Cytotoxicity Assay (luminescent)	10ml	G9290
MultiTox-Fluor Multiplex Cytotoxicity Assay (fluorescent; dual assay)	10ml	G9200
MultiTox-Glo Multiplex Cytotoxicity Assay (luminescent and fluorescent; dual assay)	10ml	G9270

Not for Medical Diagnostic Use. Additional kit formats are available.

Apoptosis Products

Product	Size	Cat.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
Caspase-Glo® 3/7 Assay	2.5ml	G8090
Caspase-Glo® 3/7 3D Assay	10ml	G8981
Caspase-Glo® 8 Assay	2.5ml	G8200
Caspase-Glo® 9 Assay	2.5ml	G8210
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792

Not for Medical Diagnostic Use. Additional kit formats are available.

4.D. Related Products (continued)

Oxidative Stress and Metabolism Assays

Product	Size	Cat.#
Cholesterol/Cholesterol Ester-Glo	5ml	J3190
Glycerol-Glo	5ml	J3150
Glucose Uptake-Glo™ Assay	5ml	J1341
Glucose-Glo™ Assay	5ml	J6021
Glutamate-Glo™ Assay	5ml	J7021
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611
Lactate-Glo™ Assay	5ml	J5021
Mitochondrial ToxGlo™ Assay	10ml	G8000
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
ROS-Glo™ H_2O_2 Assay	10ml	G8820
Triglyceride-Glo	5ml	J3160

Not for Medical Diagnostic Use. Additional kit formats are available.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Navigator System	1 each	GM2000
GloMax® Explorer System	1 each	GM3500

5. Summary of Changes

The following changes were made to the 1/23 revision of this document:

1. Related Products and legal statements were updated.
2. Font and cover image were updated.

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[a]U.S. Pat. Nos. 7,741,067, 8,361,739 and 8,603,767 and other patents and patents pending.

[b]Patent Pending.

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