



Promega

Technical Bulletin

CytoTox-Glo™ Cytotoxicity Assay

INSTRUCTIONS FOR USE OF PRODUCTS G9290, G9291 AND G9292

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CytoTox-Glo™ Cytotoxicity Assay

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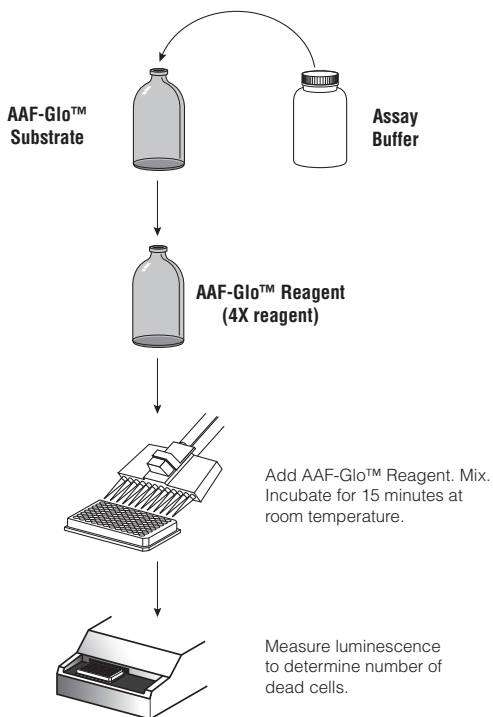
1. Description.....	1
2. Product Components and Storage Conditions	5
3. Reagent Preparation and Storage	6
4. Protocols	6
A. Determining Assay Sensitivity, Method 1.....	7
B. Determining Assay Sensitivity, Method 2.....	9
C. Example Cytotoxicity Assay Protocol and Viability (by Lysis) Assay Protocol.....	10
D. Recommended Controls.....	13
5. General Considerations.....	13
6. References	16
7. Related Products	17

1. Description

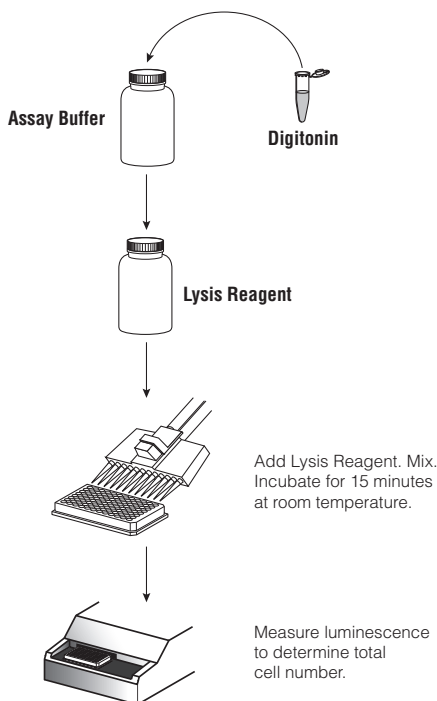
The CytoTox-Glo™ Cytotoxicity Assay^(a-c) is a single-reagent-addition, homogeneous, luminescent assay that allows measurement of the number of dead cells in cell populations (Figure 1). The CytoTox-Glo™ Assay shows excellent correlation with other methods of assessing cell viability (Figure 2). The CytoTox-Glo™ Assay measures a distinct protease activity associated with cytotoxicity (1). The assay uses a luminogenic peptide substrate (alanyl-alanyl-phenylalanyl-aminoluciferin; AAF-Glo™ Substrate) to measure “dead-cell protease activity”, which has been released from cells that have lost membrane integrity (Figure 3). The AAF-Glo™ Substrate cannot cross the intact membrane of live cells and does not generate any appreciable signal from the live-cell population. The assay selectively detects dead cells (Figure 4). The CytoTox-Glo™ Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which uses aminoluciferin as a substrate to generate a stable "glow-type" luminescent signal and is formulated to improve performance across a wide range of assay conditions.

With the addition of the Lysis Reagent (provided), the CytoTox-Glo™ Cytotoxicity Assay can also deliver the luminescent signal associated with the total number of cells in each assay well. Viability can be calculated by subtracting the luminescent signal resulting from experimental cell death from total luminescent values (Section 5).

Step 1. Measure dead-cell number.



Step 2. Measure total cytotoxicity (optional).



$$\text{Total Cell Number} - \text{Dead-Cell Number} = \text{Viable Cell Number}$$

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Figure 1. Schematic diagram of the CytoTox-Glo™ Cytotoxicity Assay.

Assay Advantages

Measure the Relative Number of Dead Cells in Culture: Measure cytotoxicity by adding a single reagent with the homogeneous “add-mix-measure” protocol.

Normalize Data for Cytotoxicity: Data normalization for dead-cell number makes results more comparable well-to-well, plate-to-plate and day-to-day.

Measure the Relative Number of Remaining Viable Cells Using a Total Lysis Protocol: Correlate increased cytotoxicity with a reduction in viable cells.

Improve Your Data: Reduce statistical probability of false positives (or negatives), and eliminate fluorescence interference issues with a stable luminescence readout.

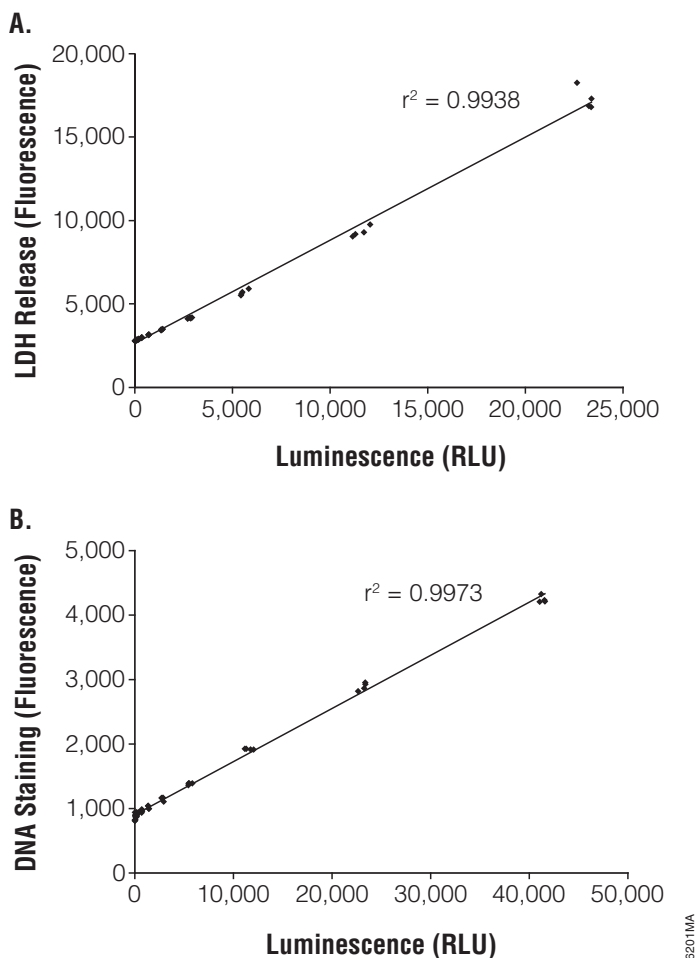


Figure 2. The CytoTox-Glo™ Cytotoxicity Assay shows strong correlation with established methods for measuring cytotoxicity. Panel A. The CytoTox-Glo™ Cytotoxicity Assay signal from serial twofold dilutions of dead cells plotted against results from the CytoTox-ONE™ Homogeneous Membrane Integrity Assay, which measures LDH release. **Panel B.** The CytoTox-Glo™ Assay signal from serial dilutions of dead cells plotted against results achieved using ethidium homodimer (DNA quantitation).

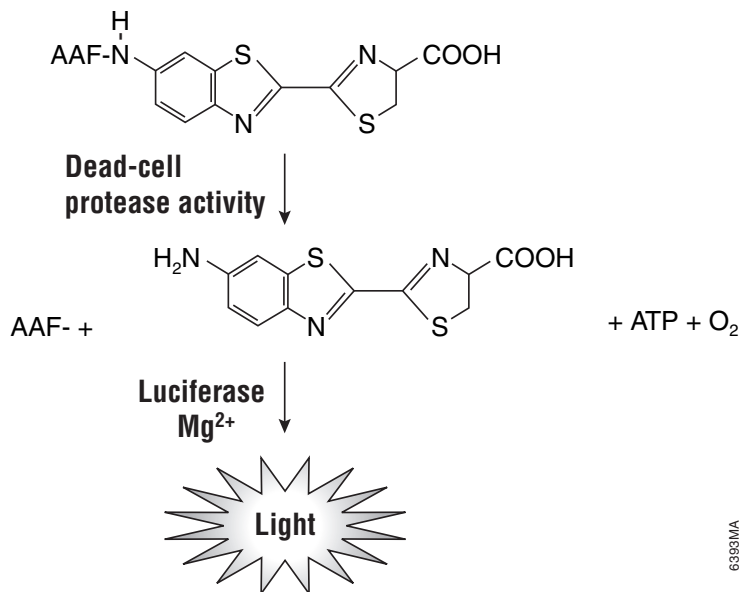


Figure 3. Cleavage of the luminogenic AAF-Glo™ Substrate by dead-cell protease activity. Following cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase-mediated production of light.

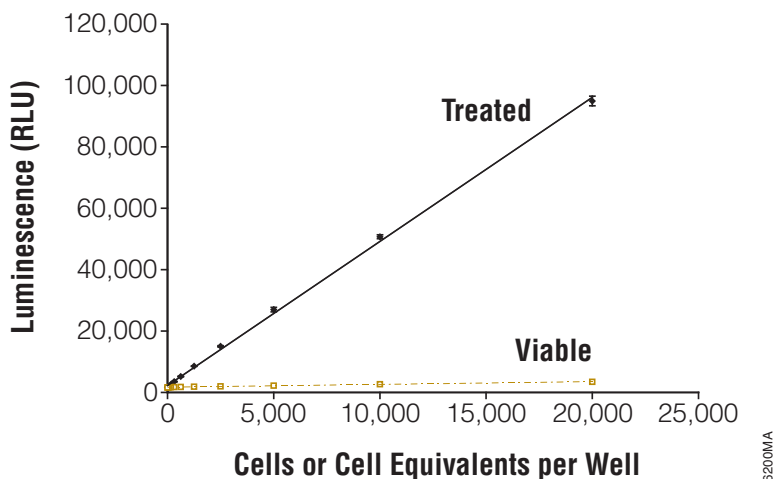


Figure 4. The CytoTox-Glo™ Cytotoxicity Assay signal derived from lysed cells is extremely sensitive and proportional to cell number and demonstrates selective detection of dead cells.

2. Product Components and Storage Conditions

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290

For Laboratory Use. G9290 contains sufficient reagents for 100 assays at 100µl per assay in a 96-well plate format or 400 assays at 25µl per assay in a 384-well plate format. Includes:

- 1 bottle AAF-Glo™ Substrate
- 2 × 5ml Assay Buffer
- 1 × 40µl Digitonin

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	5 × 10ml	G9291

For Laboratory Use. G9291 contains sufficient reagents for 500 assays at 100µl per assay in a 96-well plate format or 2,000 assays at 25µl per well in a 384-well format. Includes:

- 5 bottles AAF-Glo™ Substrate
- 10 × 5ml Assay Buffer
- 1 × 175µl Digitonin

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	2 × 50ml	G9292

For Laboratory Use. G9292 contains sufficient reagents for 1,000 assays at 100µl per assay in a 96-well plate format or 4,000 assays at 25µl per well in a 384-well format. Includes:

- 2 bottles AAF-Glo™ Substrate
- 4 × 25ml Assay Buffer
- 2 × 175µl Digitonin

Storage Conditions: Store the CytoTox-Glo™ Cytotoxicity Assay components at -20°C, protected from light. See product label for expiration date information.



3. Reagent Preparation and Storage

1. Thaw the CytoTox-Glo™ Cytotoxicity Assay components in a 37°C water bath. Mix the components to ensure homogeneity.
2. Prepare the CytoTox-Glo™ Cytotoxicity Assay Reagent by transferring the contents of one bottle of Assay Buffer to the AAF-Glo™ Substrate bottle.
Note: The appearance of the AAF-Glo™ Substrate prior to addition of Assay Buffer may vary from lot to lot. This variation does not affect assay performance.
3. Prepare the Lysis Reagent by transferring Digitonin (33µl for Cat.# G9290 and G9291; 162µl for Cat.# G9292) to the Assay Buffer (5ml for Cat.# G9290 and G9291; 25ml for Cat.# G9292). Mix well to ensure homogeneity.

Storage: The CytoTox-Glo™ Cytotoxicity Assay Reagent should be used within 12 hours if stored at room temperature. The CytoTox-Glo™ Cytotoxicity Assay Reagent (rehydrated AAF-Glo™ Substrate) and Lysis Reagent can be stored at 4°C for up to 7 days with no appreciable loss of performance.

4. Protocols

Materials to be Supplied by the User

- 96-, 384- or 1536-well, white-walled tissue culture plates compatible with luminometer and fluorometer (clear or solid bottom)
- multichannel pipettor or liquid-dispensing robot
- reagent reservoirs
- luminescence plate reader or multimode reader
- orbital plate shaker
- positive control cytotoxic compound

If you have not performed this assay with your cell line previously, we strongly recommend that you determine the assay sensitivity for your cells using one of the two methods described below (Section 4.A or 4.B). Use Method 1 to determine the linear range of the assay for your cell type. Use Method 2 to determine the practical sensitivity once you have already chosen the number of cells to use in the assay. If you do not need to determine assay sensitivity for your cells, proceed to Section 4.C.

4.A. Determining Assay Sensitivity, Method 1

In this method, the Lysis Reagent is added as the first reagent to simulate experimental cytotoxicity. Lysis Reagent is added as the first reagent to effect 100% cytotoxicity in the presence of a limited number of cells to determine assay sensitivity. The luminescence measured is total cell luminescence. In all other assay applications, Lysis Reagent is added after the CytoTox-Glo™ Reagent. CytoTox-Glo™ Reagent is first added, then luminescence is measured to determine dead cell luminescence. Lysis Reagent is then added, and luminescence is measured to determine total cell luminescence. The final concentration of the Lysis Reagent used in this method is different than that when Lysis Reagent is added after the CytoTox-Glo™ Reagent. These two concentrations of Lysis Reagent cannot be used interchangeably.

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium to remove residual trypsin and resuspend in fresh medium.

Note: For cells growing in suspension, proceed to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, then dilute to 100,000 viable cells/ml in at least 3.0ml of fresh medium.

Note: Concentrate cells by centrifugation if the cell suspension is less dense than 100,000 cells/ml.

3. Add 100µl of the 100,000 cell/ml suspension (10,000 cells/well) to all wells of row A and B in a 96-well plate.
4. Add 100µl of fresh medium to all wells in rows B-H.

4.A. Determining Assay Sensitivity, Method 1 (continued)

- Using a multichannel pipettor, mix the cell suspension in row B by pipetting, being careful not to create foam or bubbles. Transfer 100µl from each well of row B to row C. Repeat mixing, and transfer 100µl from row C to row D. Continue this process to row G. After mixing the diluted suspension in row G, aspirate 100µl from the wells, and discard. This procedure creates dilutions of 10,000 cells/well in row A to 156 cells/well in row G. Row H will serve as the no-cell background control (Table 1).

Table 1. Schematic Diagram of 96-Well Plate Layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A					10,000 Cells/Well							
B					5,000 Cells/Well							
C					2,500 Cells/Well							
D					1,250 Cells/Well							
E					625 Cells/Well							
F					313 Cells/Well							
G					156 Cells/Well							
H					0 Cells/Well							
	untreated						treated					

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- Transfer Assay Buffer (2.75ml for Cat.# G9290 and G9291; 13.75ml for Cat. G9292) to a conical tube. Prepare Lysis Reagent by transferring Digitonin (13µl for Cat.# G9290 and G9291; 65µl for Cat. G9292) to the conical tube containing Assay Buffer. Mix to ensure homogeneity.
- Using a multichannel pipettor, add 50µl of Lysis Reagent to all wells of columns 7-12 to lyse cells; these are the treated samples. Add 50µl of the remaining Assay Buffer to all wells in columns 1-6 so that all wells have equal volumes; these are the untreated samples. Mix by orbital shaking at 700-900rpm for at least 1 minute.
- Prepare the CytoTox-Glo™ Cytotoxicity Assay Reagent as described in Section 3, and add 50µl to each well. Mix briefly by orbital shaking, and incubate at room temperature for 15 minutes.

Note: Maximal sensitivity is achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 30 minutes to 1 hour, depending on plate-well density. The signal half-life is approximately 5 hours in a 96-well plate but shorter (about 2 hours) in a 1536-well plate.

9. Measure luminescence.

Note: You may need to adjust instrument gain setting (applied photomultiplier tube energy).

10. Calculate the signal-to-noise (S:N) ratios to determine practical sensitivity for your cell type for each dilution of cells (10,000 cells/well; 5,000 cells/well, etc.).

$$\text{Cytotoxicity S:N} = \frac{(\text{Average Treated RLU} - \text{Average Untreated RLU})}{\text{S.D. of No-cell Background Controls (H1 through H6)}}$$

Note: The practical level of assay sensitivity for either assay is a signal-to-noise ratio of greater than 3 standard deviations (derived from reference 2).

4.B. Determining Assay Sensitivity, Method 2

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium to remove residual trypsin and resuspend in fresh medium.

Note: For cells growing in suspension, proceed to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, then dilute to 100,000 viable cells/ml in at least 20ml of fresh medium.

Note: Concentrate the cells by centrifugation if the cell suspension is less dense than 100,000 cells/ml.

3. Divide the volume of diluted cells into two separate tubes. Subject one tube to “moderate” sonication, which is empirically determined by post-sonication morphological examination, to disrupt cell membrane integrity and to simulate a 100% population of dead cells. The second tube of untreated cells will serve as the maximum viable population.
4. Create a spectrum of viability by blending sonicated and untreated populations in 1.5ml tubes as described in Table 2.

Table 2. Spectrum of Viability Generated by Blending Sonicated and Untreated Cells.

Percent Viability	Volume of Sonicated Cells (µl)	Volume of Untreated cells (µl)
100	0	1,000
95	50	950
90	100	900
75	250	750
50	500	500
25	750	250
10	900	100
5	950	50
0	1,000	0

4.B. Determining Assay Sensitivity, Method 2 (continued)

5. After mixing each blend of sonicated and untreated cells by gentle vortexing, pipet 100µl of each blend into eight replicate wells of a 96-well plate. Add the 100% viable cell sample to column 1, 95% viable to column 2, etc. Add 100µl of cell culture medium only to column 10 to serve as the no-cell background control.
6. Add 50µl of CytoTox-Glo™ Cytotoxicity Assay Reagent to all wells, mix briefly by orbital shaking, then incubate for 15 minutes at room temperature.
Note: Maximal sensitivity is achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 30 minutes to 1 hour, depending on plate-well density. The signal half-life is approximately 5 hours in a 96-well plate but shorter (about 2 hours) in a 1536-well plate.
7. Measure luminescence.

Total Cell Number (optional)

8. Add 50µl of Lysis Reagent. Mix by orbital shaking, then incubate at room temperature for 15 minutes.
9. Measure luminescence.
10. Calculate the signal-to-noise (S:N) ratios to determine practical sensitivity for your cell type for each blend of cell viability (X = 95%, 90%, etc.).

Note: The practical level of assay sensitivity for either assay is a signal-to-

$$\text{Cytotoxicity S:N} = \frac{\text{Average luminescence for X\%} - \text{Average luminescence for 100\% viable cells}}{\text{S.D. of 100\% viable cells}}$$

$$\text{Viability S:N} = \frac{\text{Average total luminescence for X\%} - \text{Average luminescence for 0\% viable cells}}{\text{S.D. of 0\% viable cell sample}}$$

noise ratio of greater than 3 standard deviations (derived from reference 2).

4.C. Example Cytotoxicity Assay Protocol and Viability (by Lysis) Assay Protocol

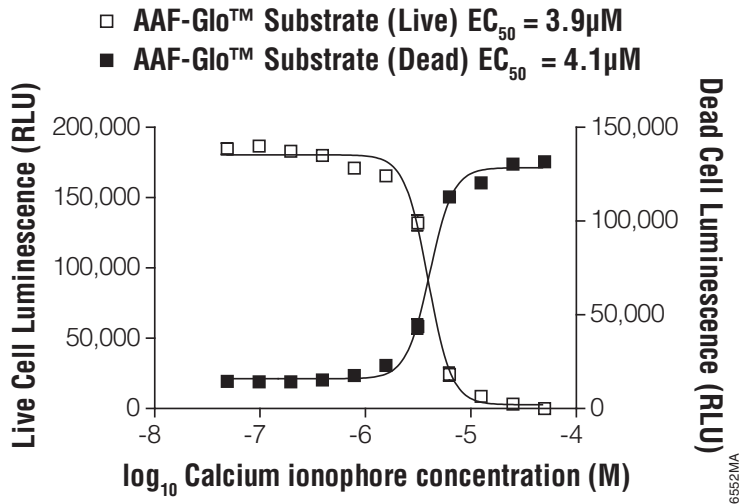


Figure 5. Determining cytotoxicity and viability by lysis. Calcium ionophore was serially diluted twofold from $100\mu\text{M}$ in RPMI 1640 medium with 10% FBS in $50\mu\text{l}$ volumes. Jurkat cells were added at 10,000 cells/well in a $50\mu\text{l}$ volume. The plate was shaken briefly using an orbital shaker, then incubated for 6 hours at 37°C in 5% CO_2 . The CytoTox-Glo™ Cytotoxicity Assay Reagent was prepared, and $50\mu\text{l}$ was added to each well. After another brief mix, the plate was incubated at room temperature for 15 minutes and the dead cell signal measured using a BMG POLARStar luminometer. The Lysis Reagent was prepared, and $50\mu\text{l}$ was added to each well to achieve complete cell lysis. Luminescence was measured after 15 minutes of incubation at room temperature. The luminescent signal collected after lysis was adjusted to reflect the “live cell” contribution by subtracting the initial dead cell signal. The data above are plotted as “dead cell” or “live cell” signal versus the concentration of calcium ionophore.

Representative data are shown in Figure 5.

1. Set up 96-well assay plates containing cells in culture medium at the desired density.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume is $100\mu\text{l}$ in each well ($25\mu\text{l}$ for a 384-well plate).

- Culture cells for the desired test exposure period.

Note: All enzymatic markers for cytotoxicity have finite activity half-lives (Figure 6). Although the protease marker(s) measured in this assay demonstrate an improved stability profile compared to other enzymatic markers under most circumstances, we recommend exposing the cells to the test compound for 24 hours or less to ensure that cytotoxicity is not underestimated. If longer exposures are desired, reducing the initial compound concentration may influence the kinetics of cytotoxicity and benefit the assessment of cell death.

- Add 50µl of CytoTox-Glo™ Cytotoxicity Assay Reagent to all wells (12.5µl for a 384-well plate). Mix briefly by orbital shaking, and incubate for 15 minutes at room temperature.

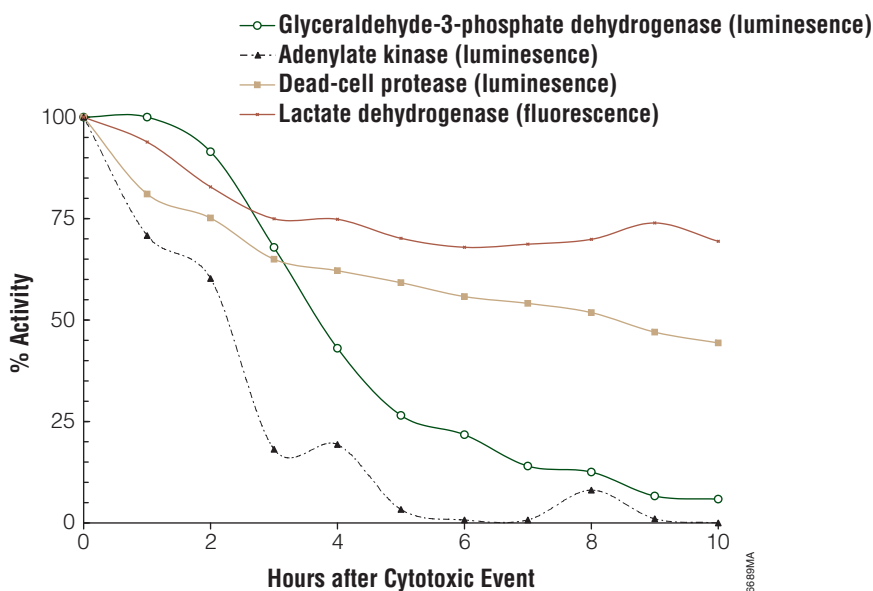


Figure 6. Half-life of enzymatic markers for cytotoxicity. Jurkat cells were plated in white-walled plates in 100µl at a density of 10,000 cells per well in RPMI 1640 medium + 10%FBS. Digitonin was added to appropriate replicate wells in 10µl (30µg/ml final concentration) every hour for 10 hours to facilitate maximal cytotoxicity. The plate was incubated at 37°C during this period. CytoTox-Glo™ Reagent and reagents to measure glyceraldehyde-3-phosphate dehydrogenase and adenylate kinase activities were prepared and added as directed by the manufacturer. Luminescence was measured after 15 minutes using a BMG POLARstar luminometer. CytoTox-ONE™ Reagent was prepared and added as directed. Fluorescence was measured using a Labsystems Fluoroskan Ascent plate reader. All data were background subtracted and plotted as a percentage of the last lysis time point.

Note: Maximal sensitivity will be achieved within 15 minutes. The signal intensity will remain largely unchanged for approximately 1 hour. The signal half-life is approximately 5 hours.

**4.C. Example Cytotoxicity Assay Protocol and Viability (by Lysis) Assay Protocol
(continued)**

5. Measure luminescence.
6. Add 50µl of Lysis Reagent to all wells (12.5µl for a 384-well plate). Mix, and incubate at room temperature for 15 minutes.
7. Measure luminescence.
8. Calculate the luminescent contribution of previously viable cells (after lysis) by subtracting the luminescent signal resulting from experimental cell death (Step 5) from total luminescence death (Step 7).

$$\text{Viable cell luminescence} = \text{Total luminescence} - \text{Experimental dead cell luminescence}$$

4.D. Recommended Controls

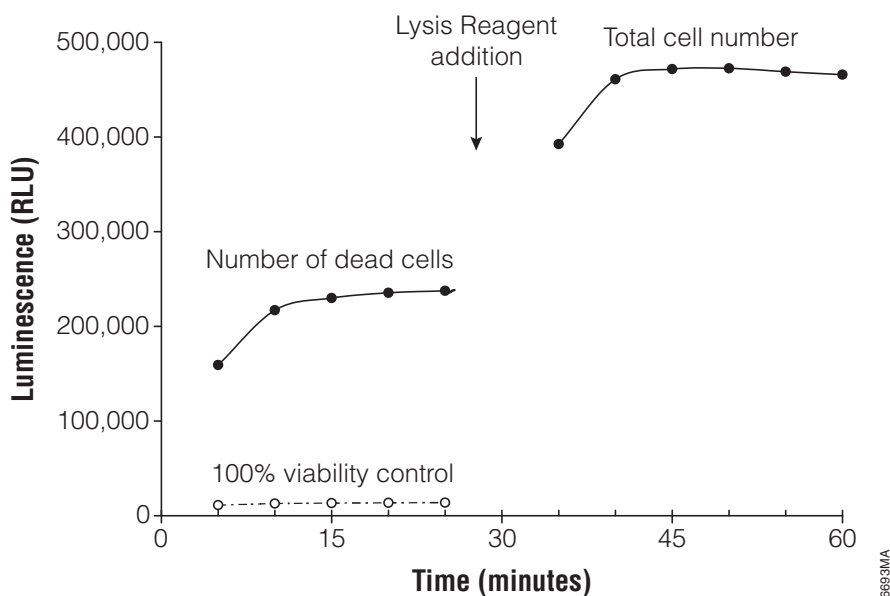


Figure 7. CytoTox-Glo™ Assay principle. Jurkat cells were washed and adjusted to 50,000 cells/ml in fresh RPMI 1640 medium + 10% FBS. The pool was split: one fraction was treated by mild sonication to cause cell death; the other was untreated. Equal volumes of treated and untreated cells were added to the same replicate wells (100µl final volume, 5,000 cell equivalents/well) to represent a sample population with 50% viable and 50% dead cells. Viability control wells received 50µl containing 2,500 untreated cells and 50µl of RPMI 1640 + 10% FBS. The AAF-Glo™ reagent was delivered to sample and control wells at 50µl per well. Luminescence was measured on a BMG POLARstar luminometer in kinetic mode for 25 minutes to collect the signal from the control and 50% dead populations. The plate was removed, and Lysis Reagent was added. After mixing by orbital shaking at 700rpm for 1 minute, luminescence from the total cytotoxicity was measured in kinetic mode for an additional 25 minutes.

No-Cell Background Control: Prepare triplicate wells without cells to serve as the negative control to determine background signal.

Untreated Cells Control: Prepare triplicate wells with untreated cells to serve as a vehicle control. Add the same solvent used to deliver the test compounds to the untreated-cells control wells.

Test Compound Control: Prepare triplicate wells without cells and containing the vehicle and test compound to test for possible interference with the CytoTox-Glo™ Assay chemistry.

Positive Control for Cytotoxicity: Prepare triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system (staurosporine, ionomycin, etc.).

5. General Considerations

Assay Principle

The CytoTox-Glo™ Assay detects both the dead and live cell populations found in a sample using sequential luminescent measures. Data are collected as relative light units (RLU). The CytoTox-Glo™ Assay achieves a signal steady-state between the dead-cell protease and firefly luciferase from 2,500 dead cells after about 15 minutes. The same number of live cells in the control well contribute a small, but measurable, background signal. After addition of digitonin to lyse remaining viable cells, a new steady-state signal is reached that represents a proportional increase in the number of dead cells (5,000 total) in the sample well (Figure 7). Therefore, cells that were initially viable contribute to the dead cell values after lysis. Because pre- and postlysis signals are stable after reaching steady-state, the viable cell contribution of any sample can be determined by a subtractive method:

Signal from Viable Cells = Total Cytotoxicity Signal - Initial Cytotoxic Signal

The Lysis Reagent has the same lytic efficiency as sonication.

Background Luminescence and Inherent Serum Activity

Tissue culture medium supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurement. The level of this protease activity can vary between different lots of serum. To correct for variability, background luminescence should be determined using samples that contain medium plus serum without cells (i.e., the no-cell background control).

5. General Considerations (continued)

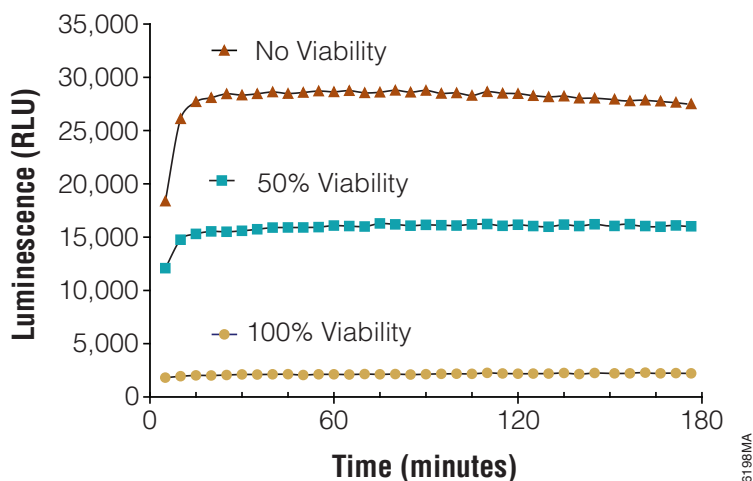


Figure 8. CytoTox-Glo™ Assay signal.

Assay Controls

In addition to a no-cell background control to establish background luminescence, we recommend including an untreated-cell control to measure maximum viability and a positive control to measure maximum cytotoxicity in the experimental design. The maximum viability control is established by adding the vehicle used to deliver the test compound to test wells. In most cases, this consists of a buffer system or medium and the equivalent amount of solvent used to deliver the test compound. Maximum cytotoxicity can be determined using the Lysis Reagent. See Section 4.A.

Temperature

The generation of luminogenic product is proportional to the protease activity of the marker associated with cytotoxicity. The activity of this protease is influenced by temperature. For best results, we recommend incubating reactions at a constant temperature to ensure uniformity across the plate. After adding the CytoTox-Glo™ Cytotoxicity Assay Reagent and mixing briefly, we suggest one of two methods:

1. Incubate at room temperature in a water-jacketed incubation module (Me'Cour, etc.).
2. Incubate at room temperature with or without orbital shaking.

Instrumentation

Luminescence mode: Luminescence chemistry does not require an external light source to generate photons but generally collects unrestricted/unfiltered light output.

Cytotoxicity Marker Half-Life

The activity of the protease marker released from dead cells has a half-life estimated to be greater than 10 hours (Figure 6). In situations where cytotoxicity occurs rapidly such as necrosis and the incubation time is greater than 24 hours, the degree of cytotoxicity may be underestimated. When using extended incubation times, adding a lytic detergent such as digitonin may be useful to determine the total cytotoxicity marker activity remaining (from remaining live cells).

Luminescent Signal Half-Life

The CytoTox Glo™ Assay chemistry uses a thermostable, recombinant luciferase to generate a “glow-type” luminescent signal that is proportional to the number of dead cells in the sample. After a short signal ramping period (0–15 minutes), the signal reaches a plateau and is relatively stable for a period of approximately 1 hour (Figure 8). Although the signal half-life is greater than 5 hours, we suggest measuring the luminescent signal at steady-state (15 minutes to 1 hour).

Light Sensitivity

Although the AAF-Glo™ Substrate demonstrates good general photostability, aminoluciferin can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

5. General Considerations (continued)

Cell Culture Medium

The AAF-Glo™ Substrate is introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment with your test compound. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of medium formulations with either very high serum content or phenol red indicator, no substantial performance differences should be observed among media.

6. References

1. Niles, A.L. *et al.* (2007) A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. *Anal. Biochem.* **366**, 197–206.
2. Zhang, J.H., Chung, T.D. and Oldenburg, K. (1999) A simple statistical parameter for use in evaluation and validation of high-throughput screening assays. *J. Bio. Mol. Screen.* **4**, 67–73.

^(a) Patent Pending.

^(b)U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294, European Pat. No. 1131441 and other patents pending.

^(c)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

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