Technical Bulletin

Caspase-Glo® 8 Assay

INSTRUCTIONS FOR USE OF PRODUCTS G8200, G8201 AND G8202.

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Caspase-Glo® 8 Assay

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1. Description

The Caspase-Glo® 8 Assay[a,b,c] is a homogeneous luminescent assay that measures caspase-8 activity. This member of the cysteine aspartic acid-specific protease (caspase) family plays a key initiator role in the extrinsic/receptor-mediated apoptotic pathway of mammalian cells (1,2). The assay provides a luminogenic caspase-8 substrate in a buffer system optimized for caspase activity, luciferase activity and cell lysis. The addition of a single Caspase-Glo® 8 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate, and generation of a "glow-type" luminescent signal (produced by the luciferase reaction; Figure 1). The signal generated is proportional to the amount of caspase activity present (Figure 2). The Caspase-Glo® 8 Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates the stable "glow-type" luminescent signal and improves performance across a wide range of assay conditions.

The Caspase-Glo® 8 Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening (HTS) of caspase-8 activity. The assay has been automated on the Beckman Coulter Biomek® 2000 and Biomek® FX Automation Workstations in both 96- and 384-well formats. Cell washing, removal of medium or multiple pipetting steps are not required (Figure 3). The caspase and luciferase enzyme activities reach a steady state so that the luminescent signal peaks rapidly and is maintained for several hours with a minimal loss of signal (Figure 4). This provides for a rapid, sensitive and flexible caspase-8 activity assay. This assay system may be used with purified enzyme preparations (Figures 2, 4, 5 and 6) or cultured cells (Figures 7, 8 and 9). The magnitude of the caspase-8 activity response in cell-based assays depends on cell type and apoptotic stimulus. This system includes an optional proteasome inhibitor (MG-132), which when added to the Caspase-Glo® 8 Reagent significantly reduces nonspecific background in cell-based assays and can improve the quality and confidence of data.

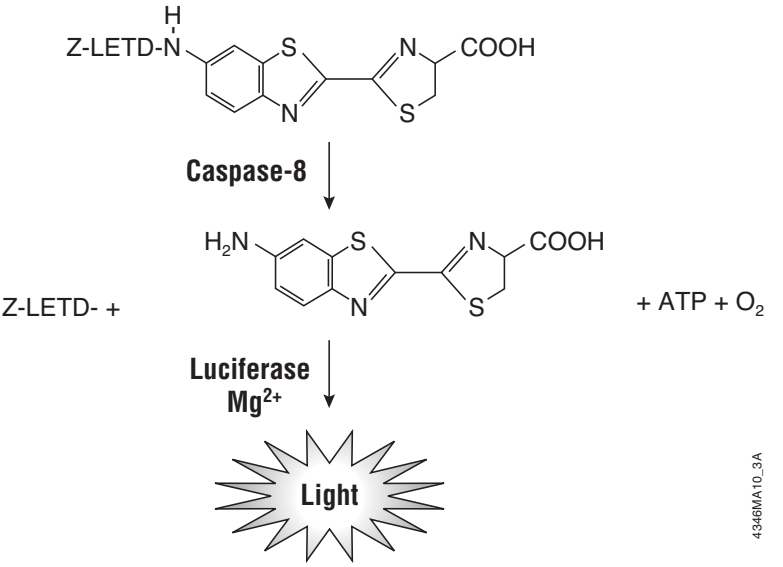

Figure 1. Caspase-8 cleavage of the luminogenic substrate containing the LETD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

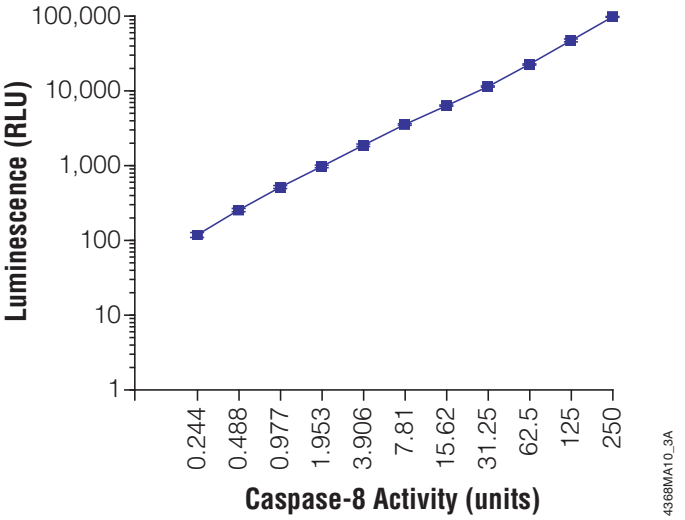

Figure 2. Luminescence is proportional to caspase-8 activity. Purified caspase-8 (BIOMOL) was diluted in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer and assayed in a total volume of 200µl per well in a 96-well plate using the Caspase-Glo® 8 Assay. Luminometer readings were taken 35 minutes after addition of the Caspase-Glo® 8 Reagent. The assay is linear over 3 orders of magnitude of caspase concentration (r^2 = 0.999, slope = 0.9435)[1]. One unit of caspase-8 is the amount of enzyme required to cleave 1pmol of substrate (Ac-LETD-pNA) per minute at 30°C, per the manufacturer's unit definition[2]. Each point represents the average of 4 wells.

[1]Determined by converting the log graph to a $\log_{10}$ linear plot to calculate r^2 and slope.

[2]Unit definitions of caspase-8 activity may vary between manufacturers; the number of units may not translate directly between vendors.

Figure 2 Notes: Due to the extended dynamic range of the Caspase-Glo® 8 Assay, data were graphed on a log scale. The number of relative light units (RLU) obtained will depend on the type of luminometer used (Section 6). For this reason, and because unit definitions of caspase activity may vary, your results may differ.

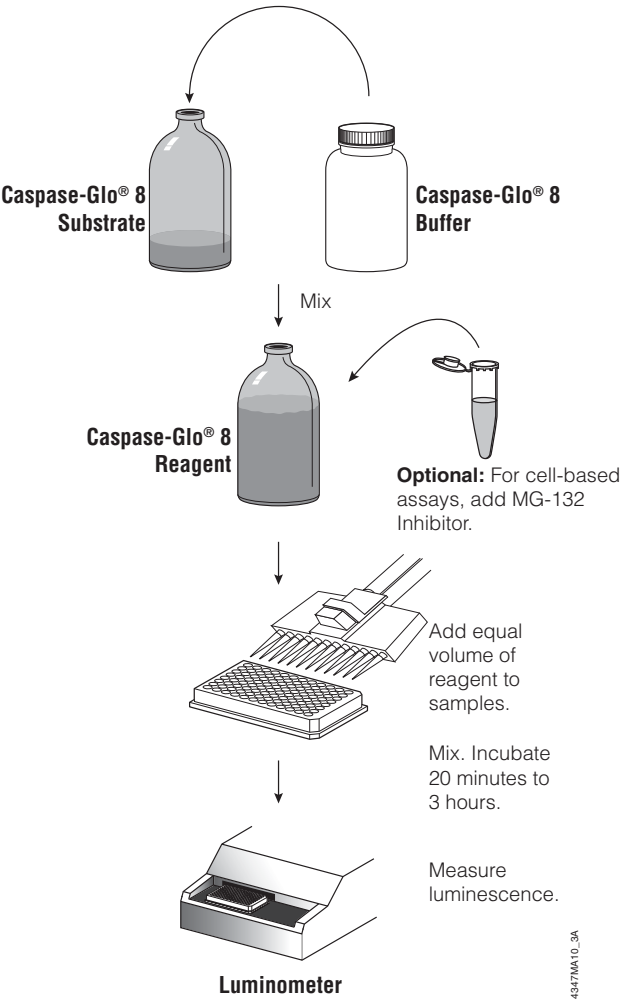

Figure 3. Schematic diagram of the Caspase-Glo® 8 Assay protocol.

Advantages of the Caspase-Glo® 8 Assay

Simplified Method: The homogeneous "add-mix-read" protocol (Figure 3) makes the assay easy to automate. The assay has been automated on the Beckman Coulter Biomek® 2000 and Biomek® FX Automation Workstations in both 96- and 384-well formats. For more information about obtaining automated protocols, see: **www.promega.com/automethods/**

Greater Sensitivity: The Caspase-Glo® Assay is more sensitive than fluorescence-based caspase assays (3,4). The luminescent assay avoids interference from inherent fluorescence signals of test compounds, providing excellent signal-to-background ratios (Figure 5). The assay sensitivity allows you to monitor apoptosis in cells at typical 96-well culture densities or use smaller quantities of purified caspase or test compounds if screening for caspase inhibitors.

Fast: There is no sample preparation or manipulation required. In contrast to fluorescence-based assays, the assay does not require extended incubation times to accumulate product to reach maximum sensitivity. Maximum sensitivity is typically reached in less than 1 hour.

Robust: The Caspase-Glo® 8 Assay delivers excellent Z´ factors with purified enzyme (Figure 5). The Z´ factor is a statistical value that compares the dynamic range of an assay to data variation in order to assess assay quality. Z´ factor values greater than 0.5 indicate excellent assay quality (5).

Amenable to Batch Processing: The extended glow signal allows plates to be read over an extended period of time (Figure 4); there is no need to use luminometers with reagent injectors.

Selective for Caspase-8: The Caspase-Glo® 8 Assay uses a luminogenic substrate containing the LETD sequence, which has been shown to be selective for caspase-8 (6,7). The assay includes an optional proteasome inhibitor (MG-132), which when added to the Caspase-Glo® 8 Reagent significantly reduces nonspecific background in cell-based assays.

Flexible: The simplified format involves addition of an equal volume of reagent to the sample and allows performing from one to thousands of assays.

Selected Citations Using the Caspase-Glo® 8 Assay

- Geiger, G.A. *et al.* (2006) Zebrafish as a "biosensor"? Effects of ionizing radiation and amifostine on embryonic viability and development. *Cancer Res.* **66**, 8172–81.

  In this study, the Caspase-Glo® 8 and Caspase-Glo® 9 Assays were used to assess caspase activation in zebrafish embryos after radiation exposure. After irradiation, caspase activation, morphological abnormalities and DNA fragmentation were observed, all three of which could be partially reversed by treatment with the radiomodifier amifostine. The authors concluded that the Caspase-Glo® Assays provided an effective and convenient means for rapidly assessing the lethal effects of radiation on Zebrafish embryos and for assaying the ability of the radiomodifier to counteract these effects.

- Hickey, T.E., Majam, G. and Guerry, P. (2005) Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytolethal distending toxin. *Infect. Immun.* **73**, 5194–7.

  In this study, the authors examined the effects of *C. jejuni* CDT (cytolethal distending toxin) on cultured monocytes. The human monocyte line 28SC was inoculated with 2µg *Campylobacter* membrane proteins per milliliter of culture. After 8 hours, the cells were harvested and assessed for caspase-8 or caspase-9 activity using the Caspase-Glo® 8 and Caspase-Glo® 9 Assays, respectively.

For additional peer-reviewed articles that cite use of the Caspase-Glo® 8 Assay, visit:
www.promega.com/citations/

2. Product Components and Storage Conditions

Product	Size	Cat.#
Caspase-Glo® 8 Assay	2.5ml	G8200

Each system contains sufficient reagents for 25 assays of 100µl per assay in 96-well plates or 100 assays of 25µl per assay in 384-well plates. Includes:

- 1 × 2.5ml Caspase-Glo® 8 Buffer
- 1 bottle Caspase-Glo® 8 Substrate (lyophilized)
- 7.5µl MG-132 Inhibitor

Product	Size	Cat.#
Caspase-Glo® 8 Assay	10ml	G8201

Each system contains sufficient reagents for 100 assays of 100µl per assay in 96-well plates or 400 assays of 25µl per assay in 384-well plates. Includes:

- 1 × 10ml Caspase-Glo® 8 Buffer
- 1 bottle Caspase-Glo® 8 Substrate (lyophilized)
- 30µl MG-132 Inhibitor

Product	Size	Cat.#
Caspase-Glo® 8 Assay	100ml	G8202

Each system contains sufficient reagents for 1,000 assays of 100µl per assay in 96-well plates or 4,000 assays of 25µl per assay in 384-well plates. Includes:

- 1 × 100ml Caspase-Glo® 8 Buffer
- 1 bottle Caspase-Glo® 8 Substrate (lyophilized)
- 300µl MG-132 Inhibitor

Storage Conditions: Store the Caspase-Glo® 8 Substrate, Caspase-Glo® 8 Buffer and MG-132 Inhibitor at –20°C protected from light. The Caspase-Glo® 8 Buffer may be thawed and stored at 4°C for 3 months or at room temperature for up to 4 days with no loss in signal. The MG-132 Inhibitor is stable for five additional freeze-thaw cycles but may be sensitive to prolonged exposure to light. See the expiration date on the product label.

3. Reagent Preparation and Storage

1. Equilibrate the Caspase-Glo® 8 Buffer and lyophilized Caspase-Glo® 8 Substrate to room temperature prior to use.
2. Transfer the contents of the Caspase-Glo® 8 Buffer bottle into the amber bottle containing Caspase-Glo® 8 Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 8 Reagent. Buffer volumes are 2.5ml for Cat.# G8200, 10ml for Cat.# G8201 and 100ml for Cat.# G8202.
3. **Optional:** To reduce nonspecific background activity in cell-based assays, MG-132 Inhibitor can be added to Caspase-Glo® 8 Reagent. Thaw the tube of MG-132 Inhibitor, mix, and add to the reagent.

   For Cat.# G8200, add 7.5µl of MG-132 Inhibitor to the 2.5ml of reagent. For Cat.# G8201, add 30µl of MG-132 Inhibitor to the 10ml of reagent. For Cat.# G8202, add 300µl of MG-132 Inhibitor to the 100ml of reagent.
4. Mix thoroughly after adding inhibitor.

   Note: The stock concentration of MG-132 Inhibitor is 20mM in DMSO. When added to the reagent, the concentration is 60µM.

Storage: The reconstituted Caspase-Glo® 8 Reagent may be stored at 4°C for up to 7 days with no loss of activity compared to that of freshly prepared reagent. Reconstituted reagent stored at 4°C for 1 week will give a signal approximately 95% of that obtained with freshly prepared reagent. When added to Caspase-Glo® 8 Reagent, the activity of the MG-132 Inhibitor is stable for 7 days when stored at 4ºC or –20ºC.

4. Detection of Caspase-8 Activity Using Purified Caspases

Materials to be Supplied by the User

- white-walled multiwell luminometer plates adequate for cell culture
- multichannel pipette or automated pipetting station
- plate shaker for mixing multiwell plates
- luminometer capable of reading multiwell plates
- purified caspase-8 enzyme (e.g., BIOMOL Cat.# SE-172)
- 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer to dilute purified enzyme
- caspase-8 inhibitor Ac-IETD-CHO (e.g., BIOMOL Cat.# P-430) if performing assays to examine caspase inhibition (Section 4.C)

Note: Caspase-specific activities and unit definitions can vary widely, depending on the manufacturer. Procedures describing the use of caspase by weight may need to be optimized, depending on the specific activity of the caspase being used.

Directions are given for performing the assay in a total volume of 200µl using 96-well plates and a luminometer. However, the assay can be easily adapted to different volumes provided the 1:1 ratio of Caspase-Glo® 8 Reagent volume to sample volume is used (e.g., 25µl of sample and 25µl of Caspase-Glo® 8 Reagent in a 384-well format).

4.A. Assay Conditions

Due to the sensitive nature of the Caspase-Glo® 8 Assay, take care to avoid contamination with solutions containing caspase enzymes or luciferin.

Prepare the following reactions to detect caspase-8 activity (or inhibition of activity) in purified enzyme preparations. "Vehicle" refers to the solvent used to dissolve the test compound or protein of interest.

- Blank reaction consisting of Caspase-Glo® 8 Reagent and vehicle
- Positive control consisting of Caspase-Glo® 8 Reagent, vehicle and purified caspase-8 enzyme
- Assays consisting of Caspase-Glo® 8 Reagent and purified caspase-8 enzyme and test compound
- Inhibition control consisting of Caspase-Glo® 8 Reagent, a known caspase-8 inhibitor and purified caspase-8 enzyme

For the inhibition control reactions, a single concentration of the known caspase-8 inhibitor can be assayed to demonstrate inhibition, or serial dilutions of the inhibitor can be assayed to determine the IC_{50} value, which can be compared to known IC_{50} values. The blank reaction is used to measure background luminescence associated with the vehicle used to deliver the test

compound in the presence of the Caspase-Glo® 8 Reagent. Luminescence for the blank reaction should be subtracted from experimental values. The positive control reaction is used to determine the maximum luminescence obtained with the purified enzyme. A positive control and a blank reaction should be performed for each plate when processing multiple assay plates.

Notes:

1. Empirical determination of the optimal caspase concentration may be necessary.
2. Use identical enzyme concentrations for the assays and the control reactions.

4.B. Standard Protocol for Purified Caspase Enzyme

1. Prior to starting the assay, prepare the Caspase-Glo® 8 Reagent as described in Section 3. Allow the reagent to equilibrate to room temperature. Mix well.
2. Prepare the blank reactions, control reactions and test samples described in Section 4.A by adding 100µl of vehicle, purified enzyme with vehicle or purified enzyme with test compound to each well of a white-walled 96-well luminometer plate.

(!) Do not touch pipet tips to the wells containing samples to avoid cross-contamination.

3. Add 100µl of Caspase-Glo® 8 Reagent to each sample. Cover the plate with a plate sealer if incubating reactions for extended periods of time (>1 hour).
4. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for the desired time. The optimal incubation period should be determined empirically.

(!) Temperature fluctuations will affect the luminescent readings (Section 6).

5. Measure the luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer.

Notes:

1. The maximal luminescent signal will be reached in approximately 20–60 minutes, and this signal will be stable for several hours (Figure 4). Peak luminescence is usually achieved sooner in assays using purified enzymes rather than cells. In general, the luminescent signal remaining at 2 hours after peak luminescence is approximately 70% of peak luminescence. The maximum recommended incubation time is 3 hours.
2. Gentle mixing may be done using an orbital plate shaker. Mixing is not required but may improve reproducibility between replicate samples.

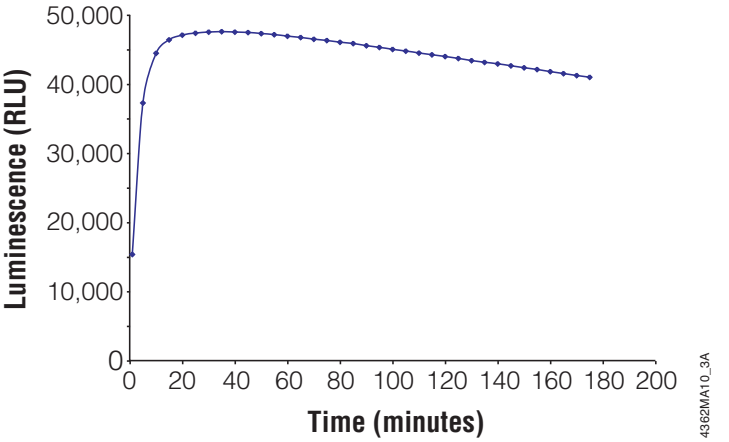

Figure 4. Signal stability using purified caspase-8 enzyme. Purified recombinant caspase-8 enzyme was assayed in a total volume of 200µl per well in a 96-well plate using the Caspase-Glo® 8 Assay. Caspase-8 was diluted to 125 units/ml in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer. One unit of caspase-8 is the amount of enzyme required to cleave 1pmol of substrate (Ac-LETD-pNA) per minute at 30°C, per the manufacturer's unit definition[1]. Each point represents the average of four wells.

[1]Unit definitions of caspase-8 activity may vary between manufacturers; the number of units may not translate directly between vendors.

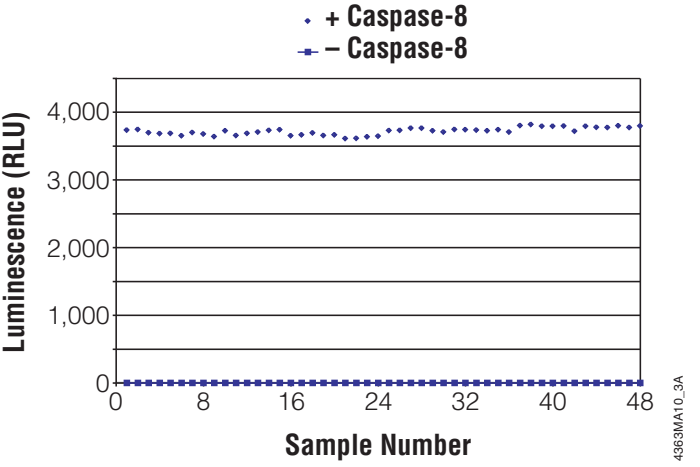

Figure 5. Z´ factor analysis. Z´ factor (5) for the Caspase-Glo® 8 Assay was calculated using recombinant caspase-8 (10 units/ml) and a no-caspase blank. Assays were performed in a total volume of 200µl in a single 96-well plate (48 wells/plate). Half of the wells of the plate contained buffer and purified caspase (+ caspase-8), and half of the wells contained buffer only with no purified caspase (– caspase-8). Z´ factor = 0.957 for this assay. One unit of caspase is the amount of enzyme required to cleave 1pmol of substrate (Ac-LETD-pNA) per minute at 30°C, per the manufacturer's unit definition.

4.C. Standard Protocol for Performing Caspase Inhibitor Studies

This protocol is intended for determining IC_{50} values of reversible inhibitors only. All IC_{50} values should be determined at luminescent signal steady state.

1. Prior to starting the assay, prepare the Caspase-Glo® 8 Reagent as described in Section 3. Allow the reagent to equilibrate to room temperature. Mix well.
2. Prepare the controls and samples described in Section 4.A in a white-walled 96-well luminometer plate; the final volume in each well should be 50µl. Add 50µl of purified enzyme or vehicle to each well, and incubate to equilibrium, typically 30 minutes at room temperature.
3. Add 100µl of Caspase-Glo® 8 Reagent to each well. Plates may be covered with a plate sealer if incubating reactions for extended periods of time (>1 hour).
4. Gently mix contents of wells at 300–500rpm using a plate shaker. Incubate at room temperature for the desired time. The optimal incubation period should be determined empirically.
5. Measure the luminescence of each sample in a plate-reading luminometer, as directed by the luminometer manufacturer.

Notes:

1. The maximal luminescent signal will be reached in approximately 20–60 minutes, and this signal will be stable for several hours (Figure 4). Peak luminescence is usually achieved sooner in assays using purified enzymes rather than cells. In general, the luminescent signal remaining at 2 hours after peak luminescence is approximately 70% of peak luminescence. The maximum recommended incubation time is 3 hours.
2. Gentle mixing may be done using an orbital plate shaker. Mixing is not required but may improve reproducibility between replicate samples.

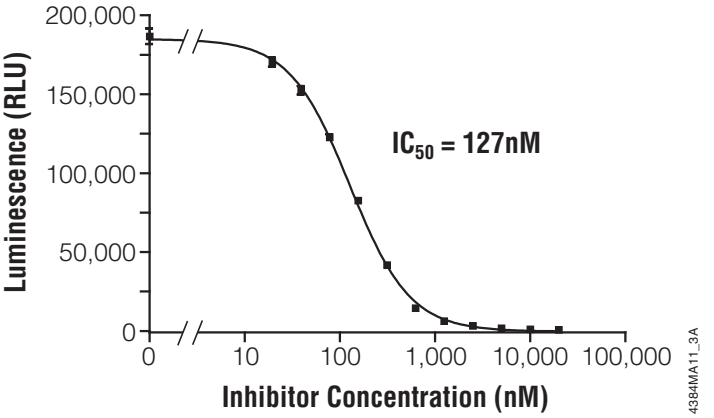

Figure 6. Determination of IC_{50} values. The IC_{50} value, the inhibitor concentration that results in 50% inhibition of maximal signal, was determined by incubating twofold serial dilutions of the inhibitor Ac-IETD-CHO with purified caspase-8 at 10 units/ml. The total volume was 100µl. The inhibitor was diluted in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer. The plate was mixed briefly on an orbital shaker, then incubated at room temperature for 30 minutes to reach steady state. An equal volume of Caspase-Glo® 8 Reagent (100µl) was added to each well, and the luminescence was measured after 1 hour.

5. Detection of Caspase-8 Activity in Cell-Based Assays

Directions are given for performing the assay in a total volume of 200µl using 96-well plates. However, the assay can be easily adapted to different volumes provided the 1:1 ratio of Caspase-Glo® 8 Reagent volume to sample volume is preserved (e.g., 75µl of sample and 75µl Caspase-Glo® 8 Reagent). The protocol includes the option of preparing Caspase-Glo® 8 Reagent to include a proteasome inhibitor (MG-132), which when added to cell-based assays significantly reduces cellular nonspecific background (Section 3).

Materials to be Supplied by the User

- white-walled multiwell plates adequate for cell culture and compatible with the luminometer being used, such as Labsystems Cliniplate
- multichannel pipette or automated pipetting station
- plate shaker, for mixing multiwell plates
- luminometer capable of reading multiwell plates

5.A. Assay Conditions

Prepare the following reactions in luminometer plates to detect caspase-8 activity in cell culture. Cells should be grown in multiwell plates that are adequate for cell culture and compatible with the luminometer being used. "Vehicle" refers to the solvent used to dissolve the test compound.

- Blank reaction consisting of Caspase-Glo® 8 Reagent and cell culture medium without cells
- No-treatment control consisting of Caspase-Glo® 8 Reagent, vehicle and cells in culture
- Assays consisting of Caspase-Glo® 8 Reagent and treated cells in culture

The blank reaction is used to measure background luminescence associated with the culture medium and Caspase-Glo® 8 Reagent. The luminescence value for the blank reaction should be subtracted from experimental values. Negative (no-treatment) control reactions are important for determining the basal protease activity of the cell culture system. The proteasome is the major contributor to background protease activity; however, some background may be contributed by the proteolytic activity of procaspase-8 (8,9). The background may account for a significant portion of the total signal. Pathway-specific positive controls with the appropriate stimuli and blank reactions should be performed for each plate when processing multiple assay plates.

Notes:

1. Empirical determination of the optimal cell number, apoptosis induction treatment and incubation time for the cell culture system may be necessary. We recommend between 10,000 and 50,000 cells/well in a 96-well plate.
2. Use identical cell numbers and volumes for the assays and control reactions.

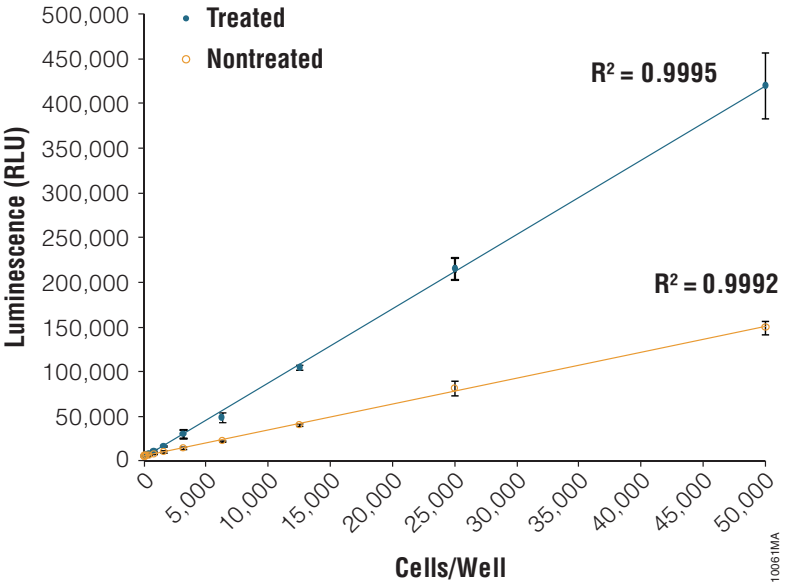

Figure 7. Sensitivity of the Caspase-Glo® 8 Assay with MG-132 Inhibitor added to the Caspase-Glo® 8 Reagent. Jurkat cells were treated with 1µg/ml soluble TRAIL (TNF-related apoptosis-inducing ligand) for 3 hours to induce apoptosis. An identical population of cells was left untreated. Equal volumes of Caspase-Glo® 8 Reagent containing MG-132 Inhibitor were added directly to cells in a 96-well plate for a final volume of 200µl per well. The assay plate was incubated at 22°C for 1 hour before recording luminescence with a GloMax®-Multi+ luminometer with Instinct™ Software. Each point represents the average of 6 wells.

5.B. Caspase-Glo® 8 Assay of Cells Cultured in a 96-Well Plate

1. Prior to starting the assay, prepare the Caspase-Glo® 8 Reagent as described in Section 3. Allow the reagent to equilibrate to room temperature. Mix well.
2. Remove 96-well plates containing cells from the incubator, and allow plates to equilibrate to room temperature.

! Do not touch pipet tips to the wells containing samples if reusing tips to avoid cross-contamination.

3. Add 100µl of Caspase-Glo® 8 Reagent to each well of a white-walled 96-well plate containing 100µl of blank, negative control cells or treated cells in culture medium. Because of the sensitivity of this assay, be careful not to touch pipet tips to the wells containing samples to avoid cross-contamination. Cover the plate with a plate sealer or lid.
Note: Lids must be removed before reading plate, Step 9.

! Temperature fluctuations will affect luminescent readings (Section 6).

4. Gently mix contents of wells using a plate shaker at 300–500rpm for 0.5–2 minutes. Incubate at room temperature for 30 minutes to 3 hours, depending upon the cell culture system.

Note: The optimal incubation time should be determined empirically. The total incubation time for the assay depends upon the culture system, but typically peak luminescent signal will be reached in 20–60 minutes. When using Caspase-Glo® 8 Reagent supplemented with MG-132 Inhibitor, peak inhibition and steady state can be delayed slightly. See Section 6 "Use of MG-132 Inhibitor in Cell-Based Assays" for a discussion on signal kinetics. The maximum recommended incubation time is 3 hours. In general, the luminescent signal remaining at 3 hours is greater than 70% of peak luminescence.

5. Measure the luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer.

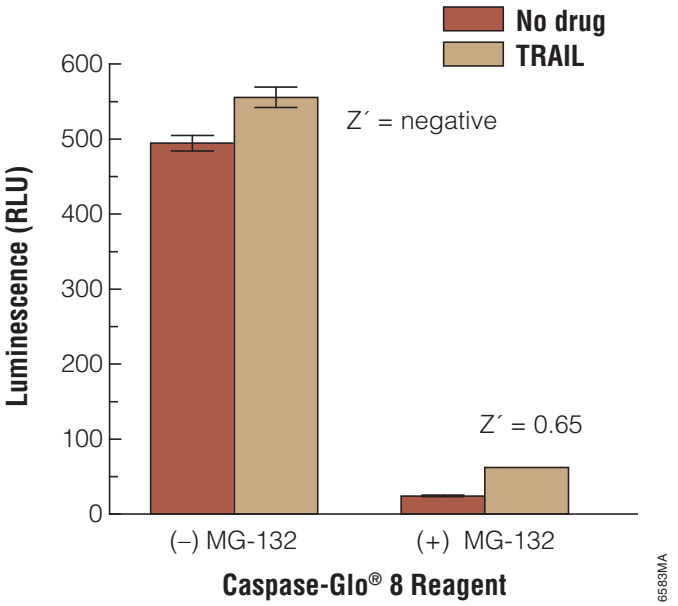

Figure 8. Improving the quality of Caspase-Glo® 8 Assay data in cell-based assays. U937 cells (15,000 cells per well cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) were plated in 90µl per well in a 96-well plate. Cells were cultured overnight at 37ºC, 5% CO_2. Soluble recombinant TRAIL (TNF-related apoptosis-inducing ligand; BIOMOL) was diluted to 10µg/ml in culture medium; 10µl was added to a portion of the plate, and 10µl of complete medium alone was added to another portion of the plate. Apoptosis was induced for 5 hours at 37ºC, 5% CO_2. The plate was allowed to equilibrate to 22ºC for 30 minutes before addition of 100µl per well of Caspase-Glo® 8 Reagent with or without MG-132 Inhibitor. Luminescence was recorded using a Dynex MLX® luminometer 1 hour after adding reagent. To demonstrate improvement in assay quality, Z´ factor values were determined with and without addition of MG-132 Inhibitor. For each reagent, 16 wells contained cells treated with complete medium alone, and 16 wells contained cells treated with TRAIL. The Z´ value for reagent without MG-132 Inhibitor was negative. When reagent was supplemented with MG-132 Inhibitor, there was a significant reduction of background, and the Z´ value improved to 0.65.

6. General Considerations

Sensitivity

In fluorescence-based assays, fluorescent substrates, such as Z-LETD-AMC or Z-LETD-AFC, are cleaved by the protease, and the released fluor exhibits a shift in the excitation/emission wavelengths. Consequently, there can be some overlap in the emission spectra of the substrate before and after cleavage, creating substantial inherent background. The luminogenic substrate (Z-LETD-aminoluciferin) is not a substrate for luciferase until cleaved to release aminoluciferin; hence there is insignificant inherent background. The low background results in a high signal:background ratio (Figure 5). The low background also allows a broad range of linearity for the assay, at least 3 orders of magnitude of caspase concentration (Figure 2); because of this, the data are typically plotted on a log-scale graph. For Jurkat cells treated with soluble TRAIL (TNF-related apoptosis-inducing ligand), the assay can detect less than 1,560 cells/well (Figure 7). We recommend using cell numbers of 10,000–50,000 cells/well in a 96-well plate or purified caspase-8 enzyme at <250 units/well to maintain maximum signal stability.

When performing assays using cells in serum-containing medium, we strongly recommend a "no-cell" blank reaction in addition to an untreated cell control to take into account the signal generated from proteolysis of the substrates in serum. The signal from the "no-cell" blank reaction can be subtracted from signal produced by the treated and untreated cells.

Unlike fluorescence-based assays, the maximum sensitivity of the Caspase-Glo® 8 Assay does not depend upon extended incubations to accumulate cleaved product. Therefore, maximum sensitivity is achieved once the caspase and luciferase activities reach steady state. Typically this occurs within 1 hour. The time required for the assay to reach steady state may vary, depending upon the cell culture system used.

Use of MG-132 Inhibitor in Cell-Based Assays

The peptide aldehyde MG-132 (Z-Leu-Leu-Leu-CHO) is a potent reversible inhibitor of the proteasome as well as calpains and cathepsins (10,11). A significant amount of nonspecific background signal in cell-based assays can be inhibited by using Caspase-Glo® 8 Reagent containing MG-132. Activity of the 20S proteasome is ATP-independent and can cleave short peptide substrates such as LETD and LEHD independent of ubiquitin targeting. Specifically, the post-glutamyl peptide hydrolytic activity (or caspase-like activity) of the proteasome is believed to be responsible for much of the signal inhibited by MG-132. MG-132 may also inhibit other proteases that are readily present following cell lysis but has minimal effect on caspase activity and signal. Figure 8 demonstrates that including MG-132 Inhibitor with the Caspase-Glo® 8 Reagent can significantly reduce nonspecific background but have only a minimal effect on the caspase response.

As shown in Figure 9, steady state and peak inhibition by MG-132 is typically achieved 30–60 minutes after adding the Caspase-Glo® 8 Reagent, depending on cell culture conditions. When using MG-132 Inhibitor, the optimal time to record luminescence is typically 1–3 hours after reagent addition.

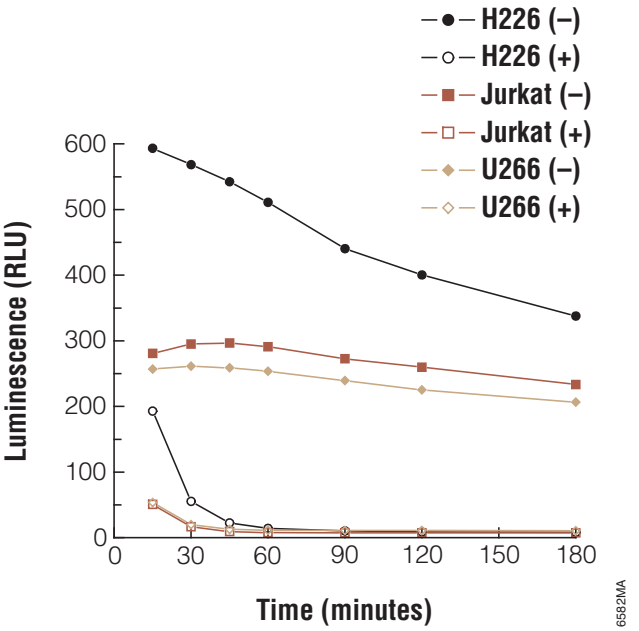

Figure 9. Kinetics of cell-based Caspase-Glo® 8 Assay with and without MG-132 Inhibitor. Signal kinetics and inhibition of nonspecific cellular background was determined using a variety of untreated cells. Jurkat and U266 cells (25,000 per well) or H226 cells (15,000 per well) were cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate and added as 100µl volumes to wells of a 96-well plate. Cells were equilibrated in a humidified 37ºC, 5% CO_2 incubator for 3 hours. Caspase-Glo® 8 Reagents were prepared with MG-132 Inhibitor (+) or with an equivalent amount of DMSO vehicle (-) and equilibrated to 22ºC for 30 minutes before use, during which time the assay plate was also equilibrated to 22°C. After reagents were added and mixed by shaking, luminescence was recorded over time using a Dynex MLX® plate luminometer, with the plate returned to a 22ºC water bath immediately after each reading. Complete culture medium alone without cells served as the background control. At each time point, the background luminescence obtained with each reagent was subtracted from each set of cell data (at 30 minutes, the average no-cell RLU values were 7.6 and 9.2 using Caspase-Glo® 8 Reagent with and without MG-132 Inhibitor, respectively). The signals obtained from the background control samples remained essentially unchanged throughout the time course.

Temperature

The intensity and rate of decay of the luminescent signal from the Caspase-Glo® 8 Assay depends on the rate of decrease in caspase activity and the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will also affect the intensity of light output and the stability of the luminescent signal. Temperature can affect 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. For batch-mode processing of multiple assay plates, include positive and negative controls for each plate. Additionally, take precautions to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require more time for equilibration than plates arranged in a single layer.

Chemicals

The chemical environment of the luciferase reaction will affect the enzymatic activity and thus luminescence intensity. Differences in luminescence intensity have been observed using different types of culture media and sera. Solvents used for various chemical compounds may affect the luciferase reaction and thus the light output from the assay. Dimethylsulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations of up to 10% in the assay and found to have a minimal effect on light output.

Luciferase Inhibitors

Any significant affect on caspase activity (i.e., luminescence) by a test compound or treatment should be verified in a secondary assay to confirm that inhibition is not due to inhibition of luciferase. True caspase inhibition can be verified using dilutions of the test compound containing 400nM Beetle Luciferin, Potassium Salt (Cat.# E1601). Add each dilution to an equal volume of the Caspase-Glo® 8 Reagent, and incubate as described in Section 4.B. A decrease in luminescence in the presence of the test compound is indicative of luciferase inhibition. The Ultra-Glo™ Luciferase used in the Caspase-Glo® Assays has been shown to be resistant to inhibition by compounds in the Library of Pharmacologically Active Compounds (LOPAC), which is often used to validate screening assays.

Due to the sensitive nature of the Caspase-Glo® 8 Assay, take care to avoid contamination of the Caspase-Glo® 8 Reagent with luciferin.

Mixing

Mixing is not required after adding the Caspase-Glo® 8 Reagent for assays with purified enzyme, although mixing may increase reproducibility between wells. We recommend mixing assays with cultured cells to aid in cell lysis.

Luminometers

For highly sensitive luminometric assays, the luminometer model and settings greatly affect the quality of the data obtained. Luminometers from different manufacturers vary in their sensitivity and dynamic range. If you are not using a GloMax® luminometer, consult the operating manual for your luminometer to determine the optimal settings. The limits should be verified on each instrument before analysis of experimental samples. The assay should be linear in some portion of the detection range of the instrument used. Within an individual luminometer there may be different gain/sensitivity settings. We recommend that you optimize the gain/sensitivity settings.

We recommend the GloMax® product family of luminometers. These instruments do not require gain adjustments to achieve optimal sensitivity and dynamic range. The Promega GloMax® family of luminometers are preloaded with Promega product protocols for ease of use. In addition, the superior detection technology built in to the instruments eliminates the need to adjust gain settings for optimal sensitivity.

7. Caspase-Glo® 8 Assays Using the Beckman Coulter Biomek® 2000 and Biomek® FX Laboratory Automation Workstations

This system has been automated on the Beckman Coulter Biomek® 2000 and Biomek® FX Automation Workstations. For more information, please see the *Automated Caspase-Glo® Assays Protocol* #EP017 at: **www.promega.com/protocols/** and refer to the documentation provided with the BioWorks™ method. Information about obtaining these methods is available at: **www.promega.com/automethods/**

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9. Related Products

Apoptosis Assays and Reagents

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	2.5ml*	G8090
Caspase-Glo® 9 Assay	2.5ml*	G8210
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml*	G7792
Caspase Inhibitor Z-VAD-FMK	50µl*	G7231
Caspase Inhibitor Ac-DEVD-CHO	100µl	G5961
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl*	G7461
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-PARP p85 Fragment pAb	50µl	G7341
DeadEnd™ Colorimetric TUNEL System	20 reactions*	G7360
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
rhTNF-α	10µg	G5241
Terminal Deoxynucleotidyl Transferase, Recombinant	300u*	M1871

*Available in additional sizes.

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10ml*	G7570
CellTiter-Blue® Cell Viability Assay (resazurin)	20ml*	G8080
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)	200 assays*	G3582
CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT)	1,000 assays*	G4000

*Available in additional sizes.

Cytotoxicity Assays

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml*	G9200
CytoTox-Fluor™ Cytotoxicity Assay	10ml*	G9260
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH)	200–800 assays*	G7890
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP (LDH)	1,000–4,000 assays	G7892
CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH)	1,000 assays	G1780

*Available in additional sizes.

Mechanism-Based Toxicity Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml*	G6320
ApoLive-Glo™ Multiplex Assay	10ml*	G6410
GSH-Glo™ Glutathione Assay	10ml*	V6911
GSH/GSSG-Glo™ Assay	10ml*	V6611

Protease Assays

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml*	G8501
DPPIV-Glo™ Protease Assay	10ml*	G8350
Proteasome-Glo™ Chymotrypsin-like Cell-Based Assay	10ml*	G8660
Proteasome-Glo™ 3-Substrate System	10ml*	G8531

*Available in additional sizes.

9. Related Products (continued)

GloMax®-Multi+ Microplate Multimode Reader with Instinct™ Software
(Requires a base unit and at least one detection unit.)

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct™ Software Base Instrument with Shaking	1 each	E8032
GloMax®-Multi+ Detection System with Instinct™ Software Base Instrument with Heating and Shaking	1 each	E9032

Detection Units for GloMax®-Multi+ Microplate Multimode Reader

Product	Size	Cat.#
GloMax®-Multi+ Luminescence Module	1 each	E8041
GloMax®-Multi+ Fluorescence Module	1 each	E8051
E8051 includes optical kits: UV (Ex: 365nm, Em: 410–460nm); Blue (Ex: 490nm, Em: 510–570nm); Green (Ex: 525nm, Em: 580–640nm); Red (Ex: 625nm, Em: 660–720nm), AFC (Ex: 405nm, Em: 495–505nm).		
GloMax®-Multi+ Visible Absorbance Module	1 each	E8061
E8061 includes filters for 450, 560, 600 and 750nm. A 490nm filter is available as an accessory.		
GloMax®-Multi+ UV-Visible Absorbance Module	1 each	E9061
E9061 includes filters for 260, 280, 450, 560, 600 and 750nm, plus two customizable filter holders.		

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

[b]U.S. Pat. Nos. 7,148,030, 7,384,758 and 7,666,987, Japanese Pat. No. 4451663 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.