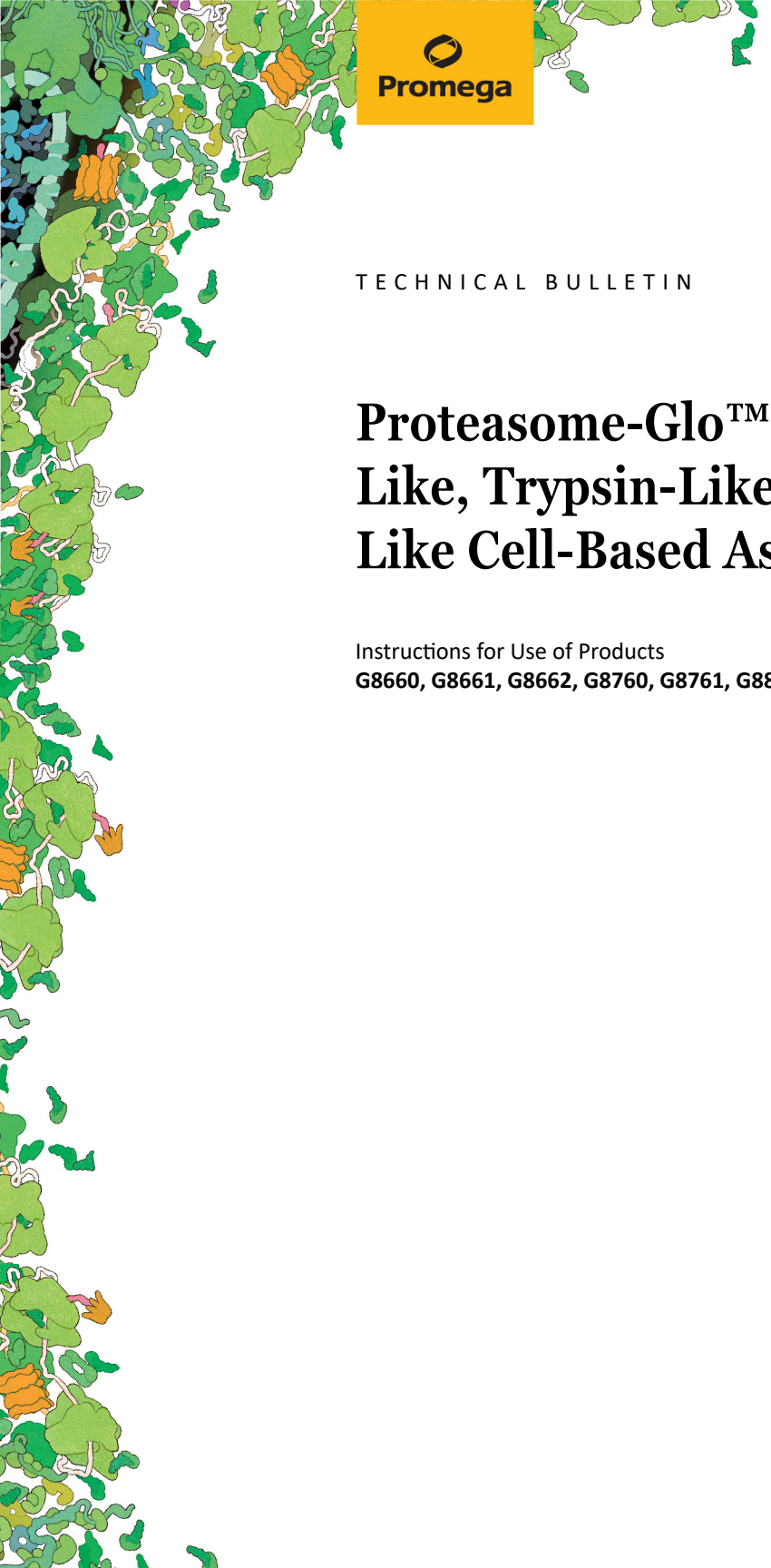

TECHNICAL BULLETIN

Proteasome-Glo™ Chymotrypsin-Like, Trypsin-Like and Caspase-Like Cell-Based Assays

Instructions for Use of Products
G8660, G8661, G8662, G8760, G8761, G8860 and G8861

Revised 3/22
TB346

Proteasome-Glo™ Chymotrypsin-Like, Trypsin-Like and Caspase-Like Cell-Based Assays

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Bulletin.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The Proteasome-Glo™ Cell-Based Assays[a] are homogeneous, luminescent assays that individually measure the chymotrypsin-like, trypsin-like or caspase-like protease activity associated with the proteasome complex in cultured cells. The 26S proteasome is a 2.5MDa multiprotein complex found both in the nucleus and cytosol of all eukaryotic cells and is comprised of a single 20S core particle and 19S regulatory particles at one or both ends (1,2). Three major proteolytic activities (described as chymotrypsin-like, trypsin-like and post-glutamyl peptide hydrolytic or caspase-like) are contained within the 20S core. Together these three activities are responsible for much of the protein degradation required to maintain cellular homeostasis including degradation of critical cell-cycle proteins, tumor suppressors, transcription factors, inhibitory proteins and damaged cellular proteins (1,3,4). Proteins destined to be degraded by the proteasome are first selectively targeted by the addition of a series of covalently attached ubiquitin molecules (4). The 19S regulatory particles are able to bind and remove the ubiquitin chain, and ATPases within the regulatory particle are thought to mediate unfolding and allow the protein to enter into the narrow core of the 20S particle, where it is degraded to yield peptides 3–25 amino acids in length (5). There is increasing interest in using highly specific proteasome inhibitors for anticancer therapy (6).

1. Description (continued)

PS-341 (bortezomib) is the first FDA-approved proteasome inhibitor for use in the treatment of certain multiple myeloma cancers, with other clinical studies in progress (7,8). Recent literature shows a growing trend to monitor all three proteasome sites as inhibitor studies focus on site preferences, characteristics and specificities (9).

The Proteasome-Glo™ Cell-Based Reagents each contain a specific luminogenic proteasome substrate in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. These peptide substrates are Suc-LLVY-aminoluciferin (Succinyl-leucine-leucine-valine-tyrosine-aminoluciferin), Z-LRR-aminoluciferin (Z-leucine-arginine-arginine-aminoluciferin) and Z-nLPnLD-aminoluciferin (Z-norleucine-proline-norleucine-aspartate-aminoluciferin) for the chymotrypsin-like, trypsin-like and caspase-like activities, respectively. The trypsin-like assay also contains two inhibitors to reduce nonspecific protease activities. Adding a single Proteasome-Glo™ Cell-Based Reagent in an "add-mix-measure" format results in proteasome cleavage of the substrate and generation of a luminescent signal produced by the luciferase reaction (Figure 1). A schematic representation of the protocol is provided in Figure 2. The Proteasome-Glo™ Reagent contains the proprietary thermostable luciferase, Ultra-Glo™ Recombinant Luciferase, and is formulated to generate a stable, "glow-type" luminescent signal that improves performance across a wide range of assay conditions. This coupled-enzyme system, with simultaneous proteasome cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that is proportional to the amount of proteasome activity in cells (Figure 3). Steady state activities of the proteasome and luciferase enzymes are reached within 5–10 minutes after adding the reagent, allowing fast and easy monitoring of activity (Figure 4).

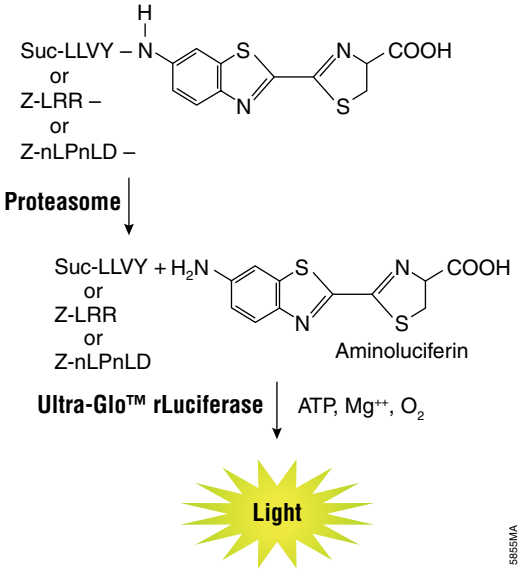

Figure 1. The luminogenic substrate containing the Suc-LLVY, Z-LRR or Z-nLPnLD sequences are recognized by the proteasome. Following cleavage by the proteasome, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to proceed and produce light.

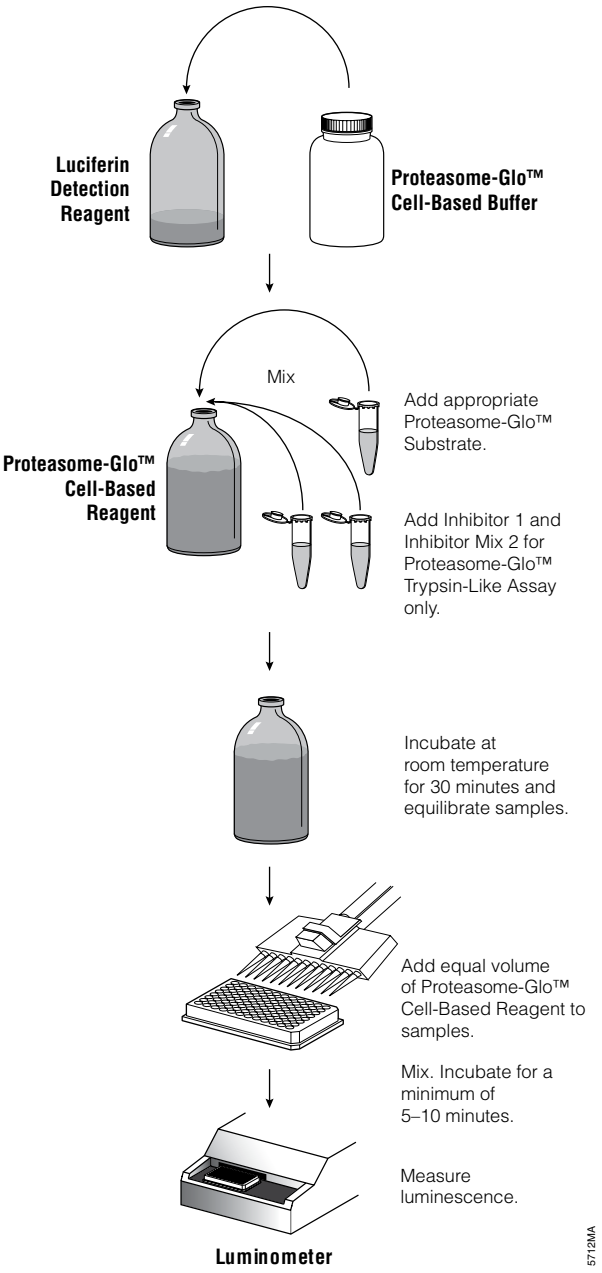

Figure 2. Schematic showing Proteasome-Glo™ Cell-Based Assay Reagent preparation and assay protocol.

1. Description (continued)

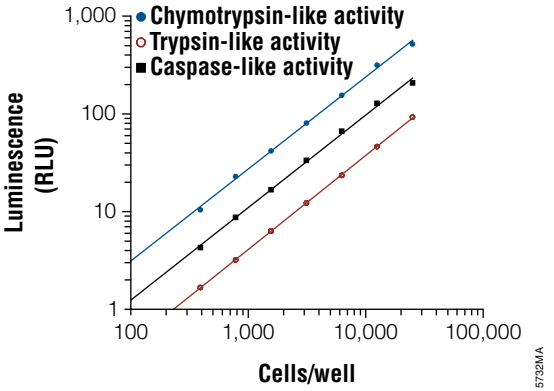

Figure 3. Luminescence is proportional to cell number. A titration of untreated U266 cells was performed in a 96-well plate using the Proteasome-Glo™ Cell-Based Assays. U266 cells (human plasma myeloma) were serially diluted in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate as 100µl/well samples. Cells were then allowed to equilibrate in a humidified 37°C, 5% CO_2 incubator for 1.5 hours. Proteasome-Glo™ Cell-Based Reagents were each prepared and equilibrated at 22°C for 30 minutes before use, during which time the assay plate was also equilibrated. Ten minutes after adding the reagent, luminescence was determined as relative light units (RLU) using a Dynex MLX® plate luminometer. Each point represents the average of four wells. The results were linear for each assay used (chymotrypsin $r^2 = 0.99$, slope = 0.94; trypsin $r^2 = 0.99$, slope = 0.96; caspase $r^2 = 0.99$, slope = 0.94). The background (no-cell control) was subtracted from each (average no-cell RLU was 3.09 for chymotrypsin, 6.54 for trypsin and 5.39 for caspase). r^2 and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot.

Assay Advantages

Simplified Method: Homogeneous "add-mix-read" protocol allows you to monitor proteasome activity directly in multiwell cell-culture plates and is easily automated.

Faster Results: Maximum sensitivity is reached 5–10 minutes after adding reagent, since the assay is not dependent on accumulation of cleaved product for sensitivity (Figure 4).

More Biologically Relevant: The assay allows convenient testing of chymotrypsin-like, trypsin-like or caspase-like proteasome activities using samples derived from cells in culture (Figure 5).

Greater Sensitivity: The coupled-enzyme format and speed of the Proteasome-Glo™ Assay result in low background, excellent signal-to-noise ratios and robust Z´-factor values (Figure 6).

Amenable to Batch Processing: The reagent formulation and Ultra-Glo™ Recombinant Luciferase generate a glow-type signal, allowing flexibility in read time once the reagent is added.

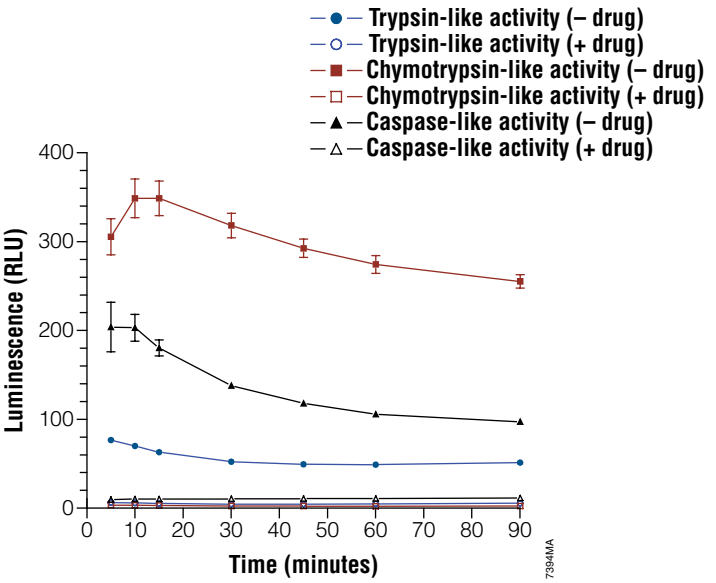

Figure 4. Proteasome signal kinetics of epoxomicin-treated cells. To demonstrate signal peak and decay, 15,000 cells/well (U266; human plasma myeloma cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) were added to a 96-well plate. Cells were equilibrated in a humidified 37°C, 5% CO_2 incubator for 2 hours prior to being treated with 0 or 8µM epoxomicin for 2 hours. Proteasome-Glo™ Cell-Based Reagents were prepared and equilibrated at 22°C for 30 minutes before use, during which time the assay plate was also equilibrated. After reagents were added and mixed by plate shaking, luminescence was recorded over time using a Dynex MLX® plate luminometer, with the plate returned to a 22°C plate incubator immediately after each reading. For each substrate, the signal from untreated cells reached its maximum at approximately 5–10 minutes and decreased over time. The signals from epoxomicin-treated cells are shown to demonstrate assay range.

1. Description (continued)

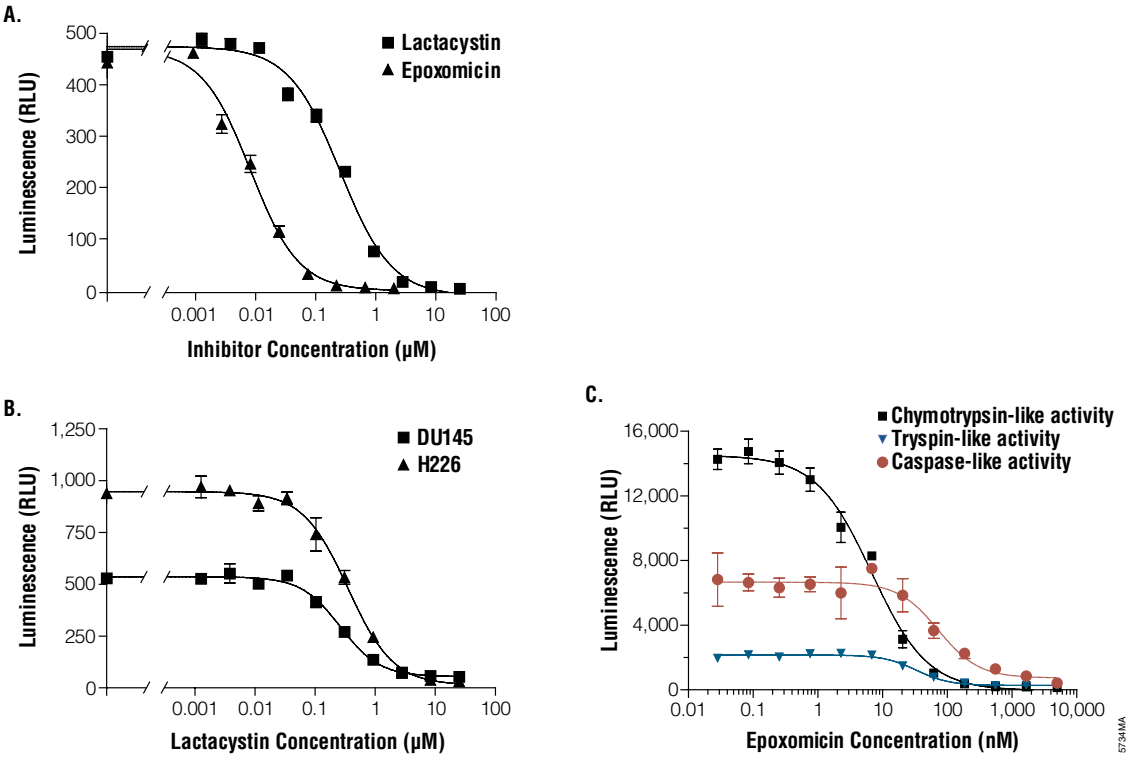

Figure 5. Bioassays using proteasome inhibitors. Panel A. Comparison of lactacystin and epoxomicin using U266 cells (human plasma myeloma). U266 cells (10,000 cells/well) were plated in 90µl/well in a 96-well plate. Cells were then equilibrated at 37°C, 5% CO_2 for 2 hours. Serial dilutions of lactacystin or epoxomicin were prepared in culture medium, and 10µl of each dilution was added to wells. The cells were incubated with the drugs for 105 minutes at 37°C, 5% CO_2. The plate was allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Chymotrypsin-Like Cell-Based Reagent was added. Luminescence was measured with a Dynex MLX® luminometer 15 minutes after adding reagent. **Panel B.** Inhibition curves using DU145 (human prostate) and H226 cells (human lung). DU145 cells (5,000 cells/well) and H226 (2,500 cells/well) were plated in 90µl/well in a 96-well plate. Cells were allowed to attach and equilibrate overnight at 37°C, 5% CO_2. Serial dilutions of lactacystin were prepared in culture medium, and 10µl of each dilution was added to wells. Cells were incubated at 37°C, 5% CO_2 for 105 minutes. The plate was removed and allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Chymotrypsin-Like Cell-Based Reagent was added. Luminescence was measured as described for Panel A. **Panel C.** Epoxomicin inhibition curves for all three protease assays in 384-well plate. U266 cells (5,000 cells/well) were plated in 20µl/well in a 384-well plate. Cells were then equilibrated at 37°C, 5% CO_2 for 2.5 hours. Serial dilutions of epoxomicin were prepared in culture medium, and 5µl of each dilution was added to wells. The cells were incubated with the drug for 2 hours at 37°C, 5% CO_2 before 25µl/well of each Proteasome-Glo™ Cell-Based Reagent was added. Luminescence was measured with a BMG Labtech FLUOstar Optima luminometer after 15 minutes.

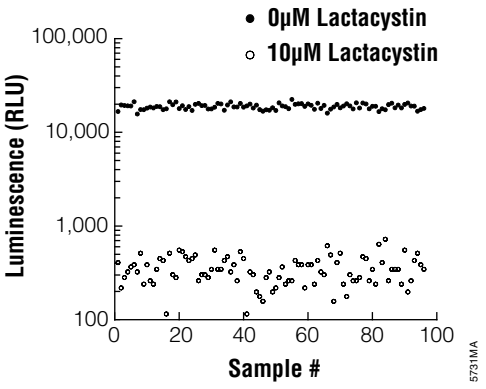

Figure 6. Z´-factor analysis in 384-well format. Z´-factor values for the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay were determined using U266 cells with and without 10µM lactacystin treatment. Cells were dispensed (1,500 cells per 5µl) using a Tecan Freedom EVO® 200 liquid handling system and allowed to equilibrate in a humidified 37ºC, 5% CO_2 incubator for 1.5 hours before lactacystin or vehicle were added in 5µl. Following a 105-minute incubation at 37ºC, 5% CO_2, the assay plate was equilibrated to ambient temperature before adding the Proteasome-Glo™ Chymotrypsin-Like Reagent (10µl/well). Luminescence was determined using a Tecan GENios Pro® luminometer. Ninety-six wells contained cells with no lactacystin added, and 96 wells contained cells treated with 10µM lactacystin. Z´-factor value = 0.77 for this assay. Z´-factor is a statistical indicator of the dynamic range and variability of assay results. A Z´-factor value of 0.5–1.0 is indicative of a high-quality assay.

Promega

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	**10ml**	**G8660**

Cat.# G8660 is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml Proteasome-Glo™ Cell-Based Buffer
- 50µl Suc-LLVY-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	**5 × 10ml**	**G8661**

Cat.# G8661 is sufficient for 500 assays at 100µl/assay in 96-well plates or 2,000 assays at 25µl/assay in 384-well plates. Includes:

- 5 × 10ml Proteasome-Glo™ Cell-Based Buffer
- 5 × 50µl Suc-LLVY-Glo™ Substrate
- 5 bottles Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	**2 × 50ml**	**G8662**

Cat.# G8662 is sufficient for 1,000 assays at 100µl/assay in 96-well plates or 4,000 assays at 25µl/assay in 384-well plates. Includes:

- 2 × 50ml Proteasome-Glo™ Cell-Based Buffer
- 2 × 250µl Suc-LLVY-Glo™ Substrate
- 2 bottles Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Trypsin-Like Cell-Based Assay	**10ml**	**G8760**

Cat.# G8760 is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml Proteasome-Glo™ Cell-Based Buffer
- 100µl Z-LRR-Glo™ Substrate
- 15µl Inhibitor 1
- 100µl Inhibitor Mix 2
- 1 bottle Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Trypsin-Like Cell-Based Assay	**5 × 10ml**	**G8761**

Cat.# G8761 is sufficient for 500 assays at 100µl/assay in 96-well plates or 2,000 assays at 25µl/assay in 384-well plates. Includes:

- 5 × 10ml Proteasome-Glo™ Cell-Based Buffer
- 5 × 100µl Z-LRR-Glo™ Substrate
- 5 × 15µl Inhibitor 1
- 5 × 100µl Inhibitor Mix 2
- 5 bottles Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Caspase-Like Cell-Based Assay	**10ml**	**G8860**

Cat.# G8860 is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml Proteasome-Glo™ Cell-Based Buffer
- 50µl Z-nLPnLD-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Caspase-Like Cell-Based Assay	**5 × 10ml**	**G8861**

Cat.# G8861 is sufficient for 500 assays at 100µl/assay in 96-well plates or 2,000 assays at 25µl/assay in 384-well plates. Includes:

- 5 × 10ml Proteasome-Glo™ Cell-Based Buffer
- 5 × 50µl Z-nLPnLD-Glo™ Substrate
- 5 bottles Luciferin Detection Reagent

Storage Conditions: Store the Proteasome-Glo™ Cell-Based Assay components at –20°C, protected from light. Each substrate may be refrozen and stored at –20°C with minimal loss of signal. The Proteasome-Glo™ Chymotrypsin-Like or Caspase-Like Reagent (combined substrate, buffer and Luciferin Detection Reagent) can be stored at 4°C or –20°C for four weeks with minimal loss of activity. The Proteasome-Glo™ Trypsin-Like Reagent is stable with minimal loss of activity for one week at 4°C or four weeks at –20°C.

3. Proteasome-Glo™ Cell-Based Reagent Preparation

1. Thaw the Proteasome-Glo™ Cell-Based Buffer, and equilibrate both buffer and lyophilized Luciferin Detection Reagent to room temperature before use.
2. Reconstitute the Luciferin Detection Reagent in the amber bottle by measuring and adding the appropriate volume of Proteasome-Glo™ Cell-Based Buffer (10ml each for Cat.# G8660, G8661, G8760, G8761, G8860 or G8861; 50ml for Cat.# G8662). The Luciferin Detection Reagent should go into solution easily in less than 1 minute.
3. Thaw the appropriate substrate and equilibrate to room temperature before use. For the Chymotrypsin-Like Assay, use the Suc-LLVY-Glo™ Substrate; for the Trypsin-Like Assay, use the Z-LRR-Glo™ Substrate; and for the Caspase-Like Assay, use the Z-nLPnLD-Glo™ Substrate. A slight precipitate may be observed. Mix well by vortexing briefly.
4. Prepare the Proteasome-Glo™ Reagent by adding the Proteasome-Glo™ Substrate to the reconstituted Luciferin Detection Reagent as per Table 1. For Proteasome- Glo™ Trypsin-Like Assay only, add Inhibitor 1 and Inhibitor Mix 2 as detailed in Table 1. After adding each inhibitor, rinse pipette tip several times.

   Note: After adding Inhibitor 1 to the resuspended Luciferin Detection Reagent, the inhibitor will appear white and cloudy; however, upon mixing the resulting solution will clarify. Label the reagent bottle to identify the substrate used. Mix to homogeneity by swirling the contents or inverting the bottle.

Table 1. Volume of Substrate and Inhibitors Added for Each Proteasome-Glo™ Cell-Based Reagent.

			Volume*		
Proteasome-Glo™ Assay	**Cat.#**	**Substrate (µM)**	**Substrate**	**Inhibitor 1**	**Inhibitor Mix 2**
Chymotrypsin-Like	G8660, G8661	Suc-LLVY-Glo™ Substrate (40µM)	50µl	—	—
	G8662	Suc-LLVY-Glo™ Substrate (40µM)	250µl	—	—
Trypsin-Like	G8760, G8761	Z-LRR-Glo™ Substrate (30µM)	100µl	15µl	100µl
Caspase-Like	G8860, G8861	Z-nLPnLD-Glo™ Substrate (40µM)	50µl	—	—

* Volume per bottle of Luciferin Detection Reagent.

5. Allow the Proteasome-Glo™ Cell-Based Reagent to stand at room temperature for 30 minutes before use. This removes any contaminating free aminoluciferin and results in lower background luminescence. Although free aminoluciferin is not detected by HPLC, it is present in trace amounts (Figure 7).

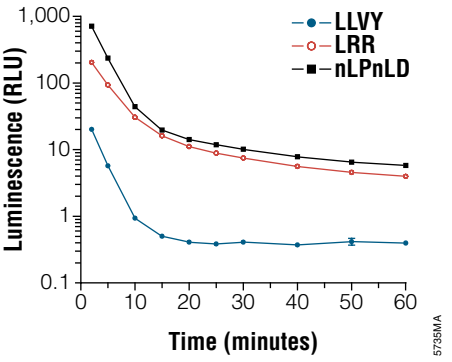

Figure 7. Time course for free aminoluciferin removal from the Proteasome-Glo™ Cell-Based Reagents. The proteasome substrate (Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin or Z-nLPnLD-aminoluciferin) were each added to reconstituted Luciferin Detection Reagent and luminescence was recorded while the plate was maintained at 22°C. Trace amounts of free aminoluciferin are present in the substrate before addition to the reagent and can be "burned-off" in a pre-incubation with the reconstituted Luciferin Detection Reagent. For maximal assay sensitivity with minimal background luminescence, the prepared Proteasome-Glo™ Cell-Based Reagents should be incubated for 30 minutes before use.

4. Protocol for Detecting Proteasome Activity from Cultured Cells

This protocol provides instructions for performing the Proteasome-Glo™ Cell-Based Assay in a total volume of 200µl using 96-well plates and a luminometer. However, the assay can be easily adapted to different volumes if the 1:1 ratio of Proteasome-Glo™ Cell-Based Reagent volume to sample volume is preserved. For example, add 25µl of sample + 25µl Proteasome-Glo™ Cell-Based Reagent for a 384-well format.

Materials to be Supplied by the User

- white-walled multiwell plates (black plates may be used, but luminescence will be reduced)
- multichannel pipette or automated pipetting station
- plate shaker for mixing multiwell plates
- plate-reading luminometer
- lactacystin, epoxomicin or other proteasome inhibitor as a control

4.A. Controls and Assay Conditions

Prepare the following reactions to detect proteasome activity (or inhibition of activity) using cells in culture:

- **Blank:** Proteasome-Glo™ Cell-Based Reagent + culture medium (without cells) and vehicle control used.
- **No-Treatment Control:** Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells and vehicle control (without test compound).

4.A. Controls and Assay Conditions (continued)

- **Inhibitor Control:** Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells with a specific proteasome inhibitor such as lactacystin or epoxomicin. See Section 5 for details.
- **Test:** Proteasome-Glo™ Cell-Based Reagent + culture medium containing cells with test compound.

The **blank** is used as a measure of background luminescence contributed by the cell-culture medium, the vehicle used to deliver test compounds, and the Proteasome-Glo™ Cell-Based Reagent and should be subtracted from all control and assay values. "Vehicle" refers to the solvent used to dissolve the inhibitor or test sample used in the study. The **no-treatment control** is used to determine the maximum luminescence obtained from untreated cells. The **inhibitor control** is used to determine the maximum inhibition of proteasome activity and helps identify nonspecific protease activity not related to the proteasome. **Test** samples represent the cells with their respective treatments.

Before You Begin

1. If you are using the Proteasome-Glo™ Trypsin-Like or Chymotrypsin-Like Cell-Based Assays and are preparing assay plates using trypsinized cells, follow this trypsinization procedure:

   Proteasome-Glo™ Trypsin-Like and Chymotrypsin-Like Assays: Recommended Trypsinization Procedure

   Following trypsinization, minute quantities of trypsin or chymotrypsin present in the resulting cell suspension used for plating and preparing assay plates will seriously affect the assay results. We recommend the following procedure to minimize this occurrence:

   a. From the parent T-75cm^2 flask of cells destined to be used, remove medium to waste, and rinse flask with D-PBS (without calcium and magnesium).

   b. Add minimal (0.5–1.0ml) amount of prewarmed trypsin:EDTA solution to flask surface and incubate until cells detach.

   c. Add 9ml of complete medium (containing serum) to cell suspension, mix, and pellet cells by gentle centrifugation.

   d. Remove medium to waste, and wash the cell pellet with 12–15ml of complete medium. Pellet cells by gentle centrifugation.

   e. Remove medium to waste, suspend cell pellet in medium, count and adjust to desired density. Cells are now ready to be plated.

2. Prepare the Proteasome-Glo™ Cell-Based Reagent as described in Section 3, and mix thoroughly before starting the assay.
3. You may need to optimize cell number and treatment duration for each cell line. For a 96-well plate format, we recommend working with approximately 10,000–20,000 suspension cells per well or 5,000–10,000 adherent cells per well.
4. For consistent results, equilibrate assay plates to a constant temperature before performing the assay.
5. Use identical cell numbers and volumes for the assays and control reactions.
6. If preparing multiple plates, replicate controls on each plate.

4.B. Proteasome-Glo™ Cell-Based Assay (96-well plate)

1. Before beginning the assay, prepare the Proteasome-Glo™ Cell-Based Reagent (see Section 3). Allow the Reagent to equilibrate to room temperature.
2. Remove the 96-well plate containing cells from the incubator, and allow the plate to equilibrate to room temperature.
3. Add 100µl of Proteasome-Glo™ Cell-Based Reagent to each 100µl of sample and appropriate controls as needed. Cover the plate using a plate sealer or lid.
4. Mix the contents of the wells at 700rpm using a plate shaker for 2 minutes. Incubate at room temperature for a minimum of 10 minutes.
5. Measure the luminescence of each sample in a plate-reading luminometer as directed by the manufacturer.

5. General Considerations

Trypsin and Chymotrypsin Can Carry Over When Using Attached Cell Lines

The Proteasome-Glo™ Trypsin-Like and Chymotrypsin-Like Cell-Based Assays are extremely sensitive at detecting trypsin and chymotrypsin protease activities, respectively. When preparing attached cell lines for plating, standard tissue culture trypsinization procedures will result in extremely high trypsin-like backgrounds and elevated chymotrypsin-like backgrounds. These elevated backgrounds occur as a result of minute amounts of trypsin/chymotrypsin carried over into the cell suspension during trypsinization and preparation of attached lines. During trypsinization and cell suspension preparation, these enzymes contaminate only the cell samples (and not the no-cell background samples); thus analysis of the final results would indicate significant cellular backgrounds that are not inhibited with epoxomicin. By following the trypsinization wash procedure described in Section 4, cell culture trypsin and contaminating trypsin/chymotrypsin activities are effectively washed out of the cell suspension used for preparing the assay and will result in assays with significantly lower backgrounds.

Sensitivity and Recommended Cell Sample Quantity

As shown in Figure 3, the Proteasome-Glo™ Cell-Based Assays have excellent sensitivity and linear range. This enhanced sensitivity occurs in part because none of the luminescent substrates are a substrate for luciferase until they are cleaved by the protease to release aminoluciferin. Pre-incubation of the substrate with the Luciferin Detection Reagent insures that any free aminoluciferin is consumed before use in the assay and results in an extremely low background. The coupled-enzyme assay is not dependent on accumulation of cleaved product because the light output is a result of luciferase consuming the aminoluciferin substrate as soon as it is produced by the protease. With the luminescent assay, maximum sensitivity is achieved as soon as the proteasome and luciferase activities reach a steady state. Typically, this occurs in 5–10 minutes; therefore, the assay is extremely sensitive within a short time frame.

Due to the sensitivity of this assay, we encourage you to use an appropriate number of cells to remain within the linear range. For a 96-well plate format, we recommend preparing bioassays to contain approximately 10,000–20,000 suspension cells per well or 5,000–10,000 adherent cells per well. Cell number can be scaled accordingly when using smaller formats. Empirical determination of the optimal cell number and treatment duration for each cell line and plate format may allow the use of even fewer cells; proteasome activity may vary significantly depending on cell type.

5. General Considerations (continued)

Assay Specificity and Controls

The Proteasome-Glo™ Cell-Based assays are formulated to minimize non-proteasome cleavage of the aminoluciferin substrates by other proteases. However, some cell lines will contain protease activity that cannot be inhibited using either epoxomicin (5–10µM for chymotrypsin-, trypsin- and caspase-like activities) or lactacystin (10µM for chymotrypsin-like activity), two readily available and highly specific inhibitors of the proteasome (7, 10–12). We recommend performing a proteasome-inhibitor control as well as an untreated-cell control in each assay plate to help define this window of activity attributable to the proteasome. Uninhibitable activity (Figure 5) is typically low and can be subtracted.

Protease Inhibitors for the Trypsin-Like Assay

The Proteasome-Glo™ Trypsin-Like Cell-Based Assay incorporates a proprietary mixture of protease inhibitors that reduce serum and cell backgrounds. These inhibitors were chosen because they have a minimal effect on proteasome activity when used at the recommended dilutions (Figure 8).

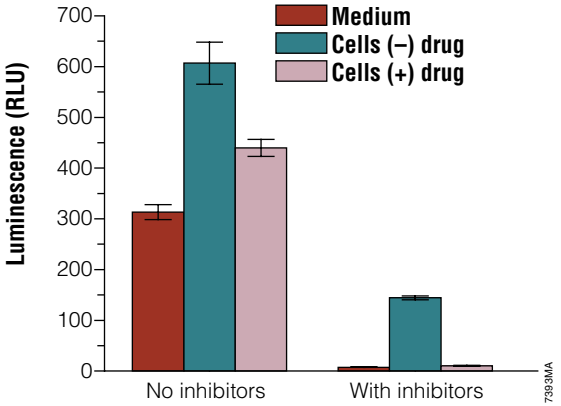

Figure 8. Specific trypsin-like measurement of the proteasome. To demonstrate the effectiveness of the protease inhibitors in Inhibitor 1 and Inhibitor Mix 2 at reducing background, 15,000 H929 cells/well (human plasma myeloma cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) or medium alone were plated in 90µl/well in a 96-well plate. Cells were cultured overnight at 37°C, 5% CO_2 before adding 0 or 5µM of epoxomicin (as 10µl/well additions) for 2 hours. Proteasome-Glo™ Trypsin-Like Reagent was prepared with and without the protease inhibitors, and following a 30-minute equilibration at 22°C, 100µl/well was added. Five minutes after adding the Proteasome-Glo™ Trypsin-Like Reagent, luminescence was determined using a Dynex MLX® plate luminometer. Proteasome-Glo™ Trypsin-Like Reagent containing the protease inhibitors reduced nonspecific LRRase background activity originating from the serum by 97%. Nonspecific cellular background activity was reduced from 43% to 2%.

Temperature and Signal Stability

Environmental factors that affect the rate of the luciferase reaction will also affect the intensity of the 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 plates, positive and negative controls should be included for each plate. Additionally, precautions should be taken to ensure complete temperature equilibration. Plates removed from a 37ºC incubator and placed in tall stacks at room temperature will require more time for equilibration than plates arranged in a single layer.

The maximal luminescent signal will typically be reached in approximately 5–10 minutes (Figure 4), and this signal will be relatively stable for 1.5 hours. Ultimate signal stability may vary depending on the cell-culture system and assay used.

Luminometers and Mixing

For highly sensitive luminometric assays such as the Proteasome-Glo™ Cell-Based Assays, the luminometer model and settings greatly affect the quality of the data obtained. Luminometers from different manufacturers will vary in their sensitivity and dynamic range. 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. Some luminometers may require different gain or sensitivity settings. We recommend that you optimize the gain or sensitivity settings. Consult your instrument operator's manual for general operating instructions. After adding the Proteasome-Glo™ Cell-Based Reagent, we recommend mixing assay plates to aid in cell permeabilization.

Note: Contamination with other luciferin-containing reagents can result in high background luminescence. Be sure that shared luminometers are cleaned thoroughly before performing this assay. Avoid workspaces and pipettes that are used with luciferin-containing solutions, including luminescence-based cell viability, apoptosis or gene reporter assays. If the interior of your luminometer is contaminated from other luciferin containing reagents and routinely introduces spurious high backgrounds, this can be overcome by applying a clear plate sealer over the wells **prior** to recording luminescence.

Chemicals

The chemical environment of the luciferase reaction will affect the enzymatic activity and thus luminescence activity. Differences in luminescence intensity have been observed using different types of media and sera. Solvents used for various chemical compounds may affect the luciferase reaction as well as the general health and responsiveness of any particular cell line (see Section 4.A). Dimethysulfoxide (DMSO), commonly used as a vehicle to solubilize organic chemicals, has been tested at final concentrations of up to 0.5% in the assay and found to have minimal effect on light output.

Promega

6. References

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7. Related Products

Protease Assays

Product	Size	Cat.#
Proteasome-Glo™ Cell-Based 3-Substrate System	3 × 50ml	G1200
Proteasome-Glo™ 3-Substrate System	50ml each	G8532
Proteasome-Glo™ Chymotrypsin-Like Assay	50ml each	G8622
Proteasome-Glo™ Trypsin-Like Assay	50ml each	G8632
Proteasome-Glo™ Caspase-Like Assay	50ml each	G8642
Calpain-Glo™ Protease Assay	50ml each	G8502
DPPIV-Glo™ Protease Assay	50ml each	G8351

Additional Sizes Available.

In Vitro Toxicology Assays

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays*	V1401
P450-Glo™ CYP2B6 Assay	50ml*	V8322
P450-Glo™ CYP1A1 Assay	50ml*	V8752
P450-Glo™ CYP1B1 Assay	50ml*	V8762
P450-Glo™ CYP1A2 Assay	50ml*	V8772
P450-Glo™ CYP2C8 Assay	50ml*	V8782
P450-Glo™ CYP2C9 Assay	50ml*	V8792
P450-Glo™ CYP3A4 Assay	50ml*	V8802
P450-Glo™ CYP2D6 Assay	50ml*	V8892
P450-Glo™ CYP2C19 Assay	50ml*	V8882
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2B6 Screening System	1,000 assays	V9781
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP3A4 Assay (Luciferin-PPXE) DMSO-Tolerant Assay	50ml*	V8912
GSH-Glo™ Glutathione Assay	10ml*	V6911

*Additional Sizes Available.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 8 Assay	100ml*	G8202
Caspase-Glo® 9 Assay	100ml*	G8212
Caspase-Glo® 3/7 Assay	100ml*	G8092
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml*	G7791
CaspACE™ Assay System, Colorimetric	100 assays*	G7220
DeadEnd™ Colorimetric TUNEL System	40 reactions*	G7130
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250

*Additional Sizes Available.

7. Related Products (continued)

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10ml*	G7570
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	200 assays*	G3582
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS)	1,000 assays*	G5421
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml*	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml*	G9200
CytoTox-Glo™ Cytotoxicity Assay	10ml*	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml*	G9260
CellTiter-Fluor™ Cell Viability Assay	10ml*	G6080

*Additional Sizes Available.

Luminometers

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

8. Summary of Changes

The following changes were made to the 3/22 revision of this document:

1. Removed discontinued Cat.# G8762 and G8862, affecting Sections 2 and 3.
2. Updated Section 7.
3. Updated disclaimers.
4. Updated cover page.

[a]European Pat. No. 1131441 and Japanese Pat. No. 4520084.