TECHNICAL BULLETIN

Proteasome-Glo™ Assay Systems

Instructions for Use of Products
G8531, G8532, G8621, G8622, G8631, G8632, G8641 and G8642

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Proteasome-Glo™ Assay Systems

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1. Description

The Proteasome-Glo™ 3-Substrate System[a,b] consists of three homogeneous bioluminescent assays that measure the three proteolytic activities associated with the proteasome (each of these three assays is also available separately). The proteasome is a multicatalytic complex in the nucleus and cytosol of all eukaryotic cells that is responsible for proteolysis of ubiquitin-tagged proteins. The catalytic core of the complex, the 20S proteasome, is a barrel-shaped assembly of 28 protein subunits that possesses three different proteolytic activities designated as chymotrypsin-like, trypsin-like and caspase-like (also termed post-glutamyl peptide hydrolase; 1,2). The catalytic sites are located on the inner surface of the central β-rings of the cylindrical particle, and access to them is controlled by narrow gated channels in the outer α-rings of the complex. The association of the 20S particle with a 19S regulatory complex at one or both ends of the barrel forms the 26S proteasome and confers an open-channel conformation, resulting in much higher rates of peptide hydrolysis (3,4). The 26S proteasome degrades polyubiquitinated proteins in an ATP-dependent manner. The 19S regulatory unit binds and removes the ubiquitin chains from tagged proteins, and ATPases within the regulatory complex appear to unfold protein substrates and translocate the unfolded polypeptides into the 20S core (2–5). The 20S catalytic core and the 19S regulatory complex are highly conserved from yeast to mammals (1). The 26S proteasome complex processes aberrant and misfolded proteins as well as proteins regulating cell cycle, growth and apoptosis and is essential for cellular function.

1. Description (continued)

The role of the proteasome in degrading several important regulatory proteins has led to the identification of the proteasome as a therapeutic target for cancer treatment. Proteasome inhibitors can induce apoptosis, and interestingly, transformed cells display greater susceptibility to proteasome inhibition than nonmalignant cells (5). The enhanced proliferative rate of malignant cells may cause accumulation of damaged proteins at a higher rate, which in turn may increase dependency on proteasomal degradation (6). The first-generation proteasome inhibitor, bortezomib (PS-341), is now an approved drug for the treatment of refractory multiple myeloma, and second-generation inhibitors are currently being developed (7).

The Proteasome-Glo™ 3-Substrate System provides three separate assays that differ in their ability to detect different protease activities based on their substrate components. The luminogenic substrates provided for the chymotrypsin-like, trypsin-like and caspase-like activities are Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnLD-aminoluciferin, respectively. Each substrate is added to a buffer system optimized for proteasome activity and luciferase activity to make a Proteasome-Glo™ Reagent for a particular catalytic activity. The individual Proteasome-Glo™ Reagent is added to test samples in an "add-mix-measure" format, resulting in proteasome-induced cleavage of the particular substrate. Substrate cleavage generates a "glow-type" luminescent signal produced by the luciferase reaction (Figure 1). In this homogeneous coupled-enzyme format, the signal is proportional to the amount of proteasome activity (Figure 2).

The Proteasome-Glo™ Reagents rely on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a "glow-type" luminescent signal and provides excellent performance across a wide range of assay conditions. The proteasome and luciferase enzyme activities reach a steady-state such that the luminescent signal peaks rapidly and is maintained for several hours with minimal loss of signal (Figure 3). The Proteasome-Glo™ Assays provide rapid, sensitive and accurate assays for the three proteolytic activities of the proteasome (Figures 4 and 5). The homogeneous Proteasome-Glo™ Assays are designed for use with multiwell-plate formats, making them ideal for automated high-throughput screening of proteasome activity and inhibition (Figure 6).

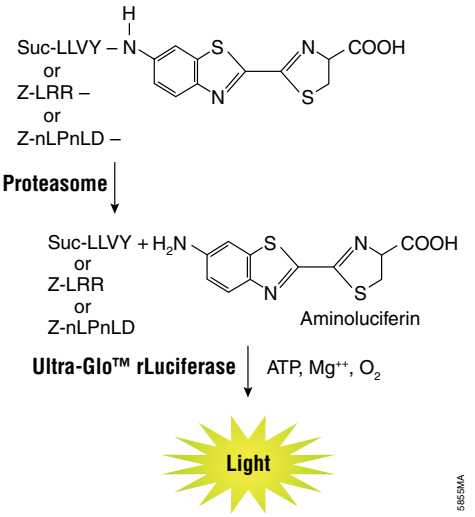

Figure 1. The luminogenic substrates containing the Suc-LLVY, Z-LRR or Z-nLPnLD sequence are recognized by the 20S proteasome. Following cleavage by the 20S proteasome, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to produce light.

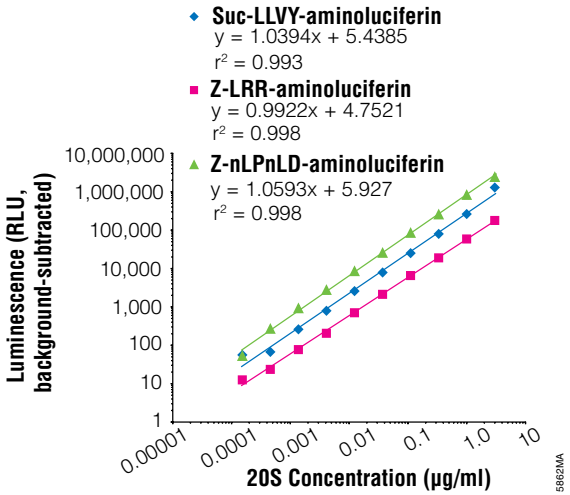

Figure 2. Luminescence is proportional to proteasome concentration. Titrations of 20S proteasome were performed in 96-well plates using the Proteasome-Glo™ 3-Substrate System. Human 20S proteasome was serially diluted in 10mM HEPES (pH 7.6). Thirty minutes after adding the individual Proteasome-Glo™ Reagents separately, luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer. Results were linear over 4 logs of 20S proteasome concentration for all three assays. Each point represents the average of four wells. The background (blank without 20S) was subtracted from each. Values for r^2 and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot.

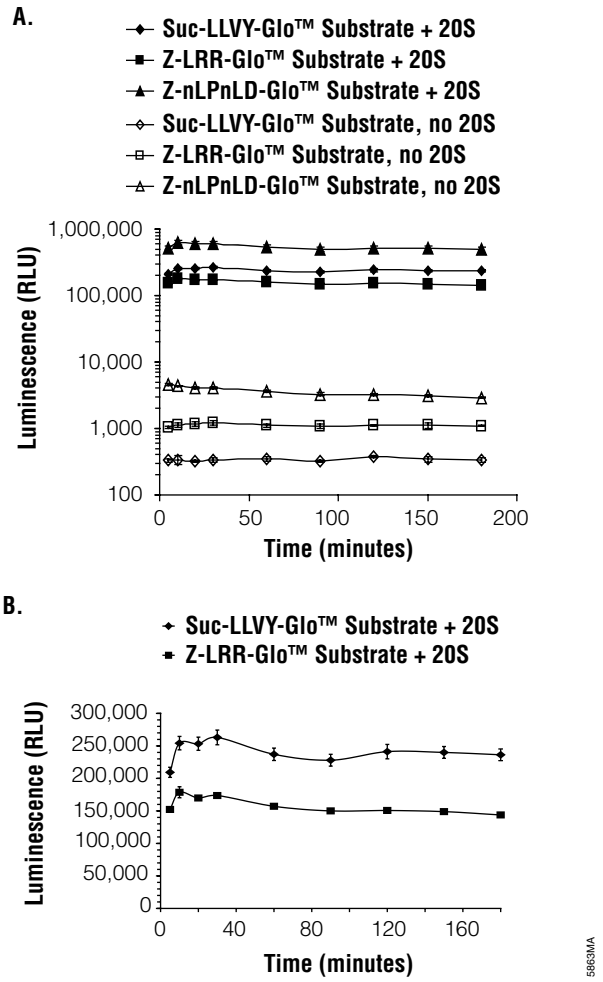

Figure 3. Signal stability of the Proteasome-Glo™ Assay Systems. Human purified 20S proteasome (1µg/ml) was assayed in 96-well plates using the individual Proteasome-Glo™ Assays. Luminescence was monitored at various time points over 3 hours on a GloMax® 96 Microplate Luminometer. **Panel A.** The signals peak rapidly and then are very stable for all three assays as shown on a log scale. **Panel B.** The stable signals generated with 20S proteasome are shown for the chymotrypsin-like (Suc-LLVY-Glo™) and trypsin-like (Z-LRR-Glo™) Substrates on a linear scale.

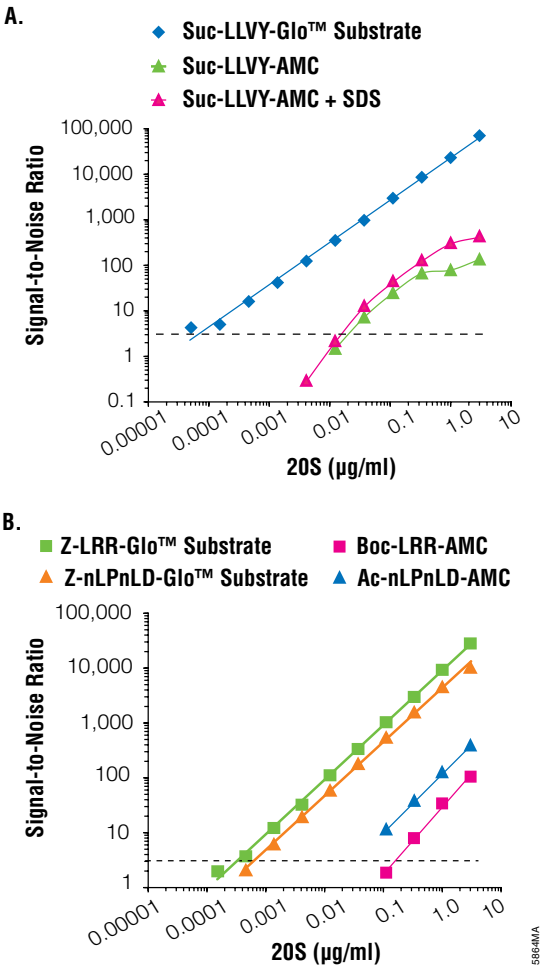

Figure 4. Sensitivity of the Proteasome-Glo™ Assays compared to fluorescent assays. Human purified 20S proteasome was titrated and assayed in 96-well plates using the Proteasome-Glo™ 3-Substrate System or comparable fluorogenic substrates. Luminescence and fluorescence were monitored at 30 minutes on a GloMax® 96 Microplate Luminometer or a Labsystems Fluoroskan Ascent plate reader, respectively. Results are plotted as signal-to-noise ratios. The limit of detection is defined as the amount of 20S proteasome giving a signal-to-noise ratio >3 (dashed lines). **Panel A.** The Proteasome-Glo™ Chymotrypsin-Like Assay (Suc-LLVY-Glo™ Substrate) was compared to a Suc-LLVY-AMC substrate. The fluorescent assay was performed with or without 0.02% SDS. **Panel B.** The Proteasome-Glo™ Trypsin-Like Assay (Z-LRR-Glo™ Substrate) was compared to the fluorogenic substrate, Boc-LRR-AMC, and the Proteasome-Glo™ Caspase-Like Assay (Z-nLPnLD-Glo™ Substrate) was compared to the fluorogenic substrate, Ac-nLPnLD-AMC. The signal-to-noise ratios are greater, and the limits of detection are significantly lower for all of the luminescent proteasome assays compared to the fluorescent proteasome assays.

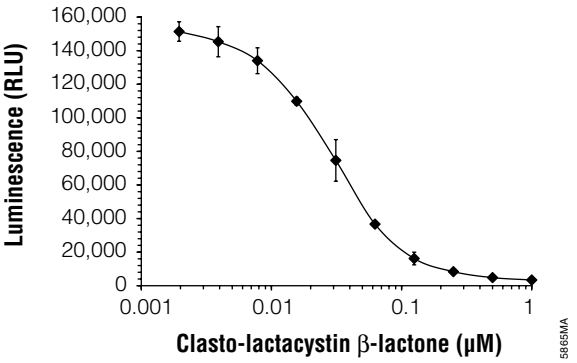

Figure 5. Determination of IC_{50} values. The inhibitor concentration that results in 50% inhibition (IC_{50}) was determined for the proteasome irreversible inhibitor, clasto-lactacystin β-lactone (8), using the Proteasome-Glo™ Chymotrypsin-Like Assay. The inhibitor was resuspended in DMSO, serially diluted in 10mM HEPES (pH 7.6) and combined with 1µg/ml 26S proteasome (Biomol) in 96-well plates. Reagent containing the Suc-LLVY-Glo™ Substrate was added after one hour, and luminescence was recorded 10 minutes after reagent addition. GraphPad Prism® software was used to calculate the IC_{50} value, which was 30nM.

Assay Advantages

Broad Dynamic Range: The assays are linear over 4 logs of proteasome concentrations and can detect 20S proteasome at concentrations as low as 0.5ng/ml (1pM) (Figures 2 and 4).

Fast: Maximum sensitivity is reached in 10–30 minutes after adding reagent (Figure 3) because the assays are not dependent on accumulation of cleaved product for sensitivity.

Greater Sensitivity: The coupled-enzyme format and the speed of the Proteasome-Glo™ Assay results in low background and excellent signal-to-noise ratios. The assays are significantly more sensitive than fluorescence-based proteasome assays (Figure 4).

Accurate: The broad linear range and excellent sensitivity readily translate to accurate kinetic analysis of inhibitors (Figure 5).

Simplified Method: The "add-mix-read" protocols make the assays amenable to automation (Figure 6).

Amenable to Batch Processing: The coupled-enzyme, homogeneous format results in a stable, glow-type signal, allowing flexibility in read time once the reagent is added (Figure 3).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ 3-Substrate System	**10ml**	**G8531**

Includes 3 individual kits. Each kit contains sufficient reagent for 100 assays at 100µl/assay or 200 assays at 50µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 1 kit Proteasome-Glo™ Chymotrypsin-Like Assay (Cat.# G8621)
- 1 kit Proteasome-Glo™ Trypsin-Like Assay (Cat.# G8631)
- 1 kit Proteasome-Glo™ Caspase-Like Assay (Cat.# G8641)

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ 3-Substrate System	**50ml**	**G8532**

Includes 3 individual kits. Each kit contains sufficient reagent for 500 assays at 100µl/assay or 1,000 assays at 50µl/assay in 96-well plates or 2,000 assays at 25µl/assay in 384-well plates. Includes:

- 1 kit Proteasome-Glo™ Chymotrypsin-Like Assay (Cat.# G8622)
- 1 kit Proteasome-Glo™ Trypsin-Like Assay (Cat.# G8632)
- 1 kit Proteasome-Glo™ Caspase-Like Assay (Cat.# G8642)

Items Available Separately

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Chymotrypsin-Like Assay	**10ml**	**G8621**

Includes:

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

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Chymotrypsin-Like Assay	**50ml**	**G8622**

Includes:

- 50ml Proteasome-Glo™ Buffer
- 1 bottle Luciferin Detection Reagent
- 250µl Suc-LLVY-Glo™ Substrate

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Trypsin-Like Assay	**10ml**	**G8631**

Includes:

- 10ml Proteasome-Glo™ Buffer
- 1 bottle Luciferin Detection Reagent
- 100µl Z-LRR-Glo™ Substrate

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Trypsin-Like Assay	**50ml**	**G8632**

Includes:

- 50ml Proteasome-Glo™ Buffer
- 1 bottle Luciferin Detection Reagent
- 500µl Z-LRR-Glo™ Substrate

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Caspase-Like Assay	**10ml**	**G8641**

Includes:

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

PRODUCT	SIZE	CAT.#
Proteasome-Glo™ Caspase-Like Assay	**50ml**	**G8642**

Includes:

- 50ml Proteasome-Glo™ Buffer
- 1 bottle Luciferin Detection Reagent
- 250µl Z-nLPnLD-Glo™ Substrate

Storage Conditions: Store the Proteasome-Glo™ Assays at –20°C protected from light. The Proteasome-Glo™ Buffer may be thawed and stored at 4°C for 2 months with no loss in signal. The Proteasome-Glo™ Substrates may be refrozen and stored at –20°C with minimal loss of signal. Proteasome-Glo™ Reagent (combined Proteasome-Glo™ Substrate, Proteasome-Glo™ Buffer and Luciferin Detection Reagent) can be stored at 4°C or –20°C for 1 month with minimal loss of activity.

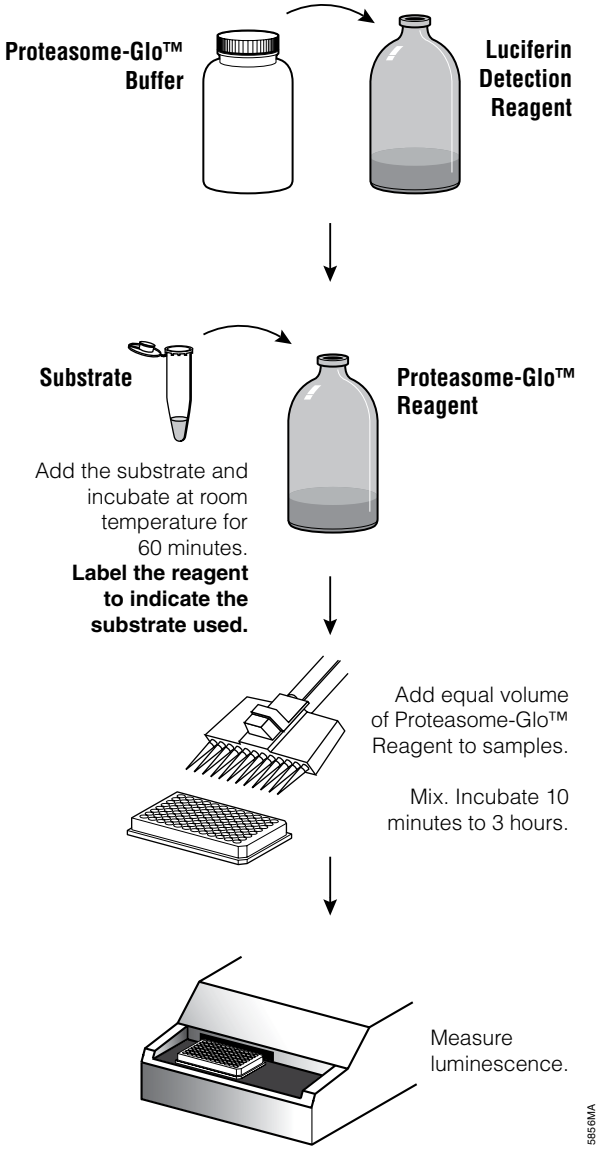

Figure 6. Flow diagram showing preparation and use of the Proteasome-Glo™ Reagent.

3. Reagent Preparation

Directions are given for performing the Proteasome-Glo™ Assays in a total volume of 100µl using 96-well plates and a luminometer. However, the assays can be easily adapted to different volumes if a 1:1 ratio of Proteasome Glo™ Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl Proteasome-Glo™ Reagent in a 384-well format).

Materials to be Supplied by the User

- white-walled multiwell plates (black plates may be used, but RLU will be reduced)
- multichannel pipette or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- 20S proteasome enzyme (e.g., Biomol Cat.# PW8720 or Boston Biochem Cat.# E-360)
- 10mM HEPES Buffer (pH 7.6, for proteasome dilution)

Proteasome-Glo™ Reagent Preparation

1. Thaw the Proteasome-Glo™ Buffer, and equilibrate both the buffer and the lyophilized Luciferin Detection Reagent to room temperature before use.
2. Reconstitute the Luciferin Detection Reagent in the amber bottle by adding the appropriate volume of Proteasome-Glo™ Buffer (10ml for Cat.# G8621, G8631, G8641; 50ml for Cat.# G8622, G8632, G8642). The Luciferin Detection Reagent should go into solution easily in less than one 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; 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 resuspended Luciferin Detection Reagent as per Table 1. **Label the reagent bottle to identify the substrate used.**
5. Allow the Proteasome-Glo™ Reagent to sit at room temperature for 60 minutes before use. This allows the removal of any contaminating free aminoluciferin. Although free aminoluciferin is not detected by HPLC, it is present in trace amounts (Figure 7).

   Note: The Protoeasome-Glo™ Reagent (combined Proteasome-Glo™ Substrate, Proteasome-Glo™ Buffer and Luciferin Detection Reagent) can be stored at 4°C or –20°C for 1 month with minimal loss of activity.

Table 1. Volume of Substrate Added to Create Proteasome-Glo™ Reagent.

Proteasome-Glo™ Assay	Cat.#	Substrate	Volume of Substrate Added	Substrate Concentration in Reagent
Chymotrypsin-Like Assay	G8621	Suc-LLVY-Glo™	50µl	40µM
Chymotrypsin-Like Assay	G8622	Suc-LLVY-Glo™	250µl	40µM
Trypsin-Like Assay	G8631	Z-LRR-Glo™	100µl	30µM
Trypsin-Like Assay	G8632	Z-LRR-Glo™	500µl	30µM
Caspase-Like Assay	G8641	Z-nLPnLD-Glo™	50µl	40µM
Caspase-Like Assay	G8642	Z-nLPnLD-Glo™	250µl	40µM

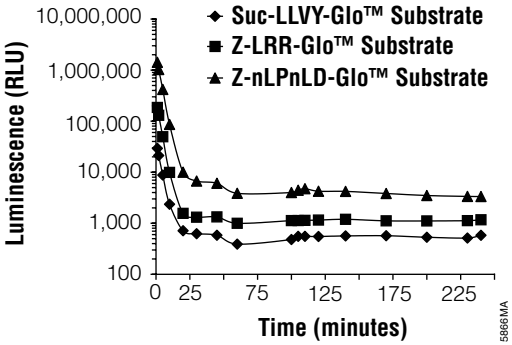

Figure 7. Time course of free aminoluciferin removal from the Proteasome-Glo™ Reagents. The proteasome substrates (Suc-LLVY-Glo™, Z-LRR-Glo™ and Z-nLPnLD-Glo™ Substrates) were added to the individual bottles of reconstituted Luciferin Detection Reagent, and a time course of luminescence loss was recorded. Trace amounts of free aminoluciferin are present in the substrate and are removed by incubation with the reconstituted Luciferin Detection Reagent. To achieve maximal assay sensitivity with minimal background luminescence, the prepared Proteasome-Glo™ Reagent should be incubated for 60 minutes at room temperature before use.

4. Assay for Detection of Proteasome Activity

4.A. Assay Conditions

Prepare the following reactions to detect proteasome activity (or inhibition of activity) in purified enzyme preparations:

- **Blank:** Proteasome-Glo™ Reagent + vehicle control for test compound or inhibitor, if used.
- **Positive Control:** Proteasome-Glo™ Reagent + vehicle control + purified proteasome enzyme (20S or 26S).
- **Assay:** Proteasome-Glo™ Reagent + test compound + purified proteasome enzyme (20S or 26S).

The blank is used as a measure of any background luminescence associated with the test compound vehicle and Proteasome-Glo™ Reagent and should be subtracted from experimental values. The positive control is used to determine the maximum luminescence obtainable with the purified enzyme system. Vehicle refers to the solvent used to dissolve the inhibitor or test compound used in the study.

Notes:

1. Prepare the Proteasome-Glo™ Reagent as described in Section 3 and mix thoroughly before starting the assay.
2. The final concentration of proteasome should be within the linear range of the assay (Figure 2).
3. The recommended proteasome dilution buffer is 10mM HEPES (pH 7.6).
4. Use identical enzyme concentrations for the assay and positive control reactions.
5. For gentle mixing you may use a plate shaker.
6. The maximal luminescent signal will be reached in 10–30 minutes and will have a half-life of several hours (Figure 3).
7. **Do not use SDS as an activating agent for the assay.** Although a low percentage of SDS is frequently used to monitor the chymotrypsin-like activity of the proteasome, it is not necessary for activation in this luminescent assay and is detrimental to the luciferase.

4.B. Standard Assay (96-well, 100µl Final Reaction Volume)

1. Add 50µl of Proteasome-Glo™ Reagent to each well of a white 96-well plate containing 50µl of blank, control or test sample. If reusing tips, be careful not to touch pipette tips to the wells containing samples to avoid cross-contamination.
2. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for 10 minutes to 3 hours depending upon convenience of reading time (Figure 3, Panel A). Maximal signal is reached typically within 10–30 minutes using purified 20S proteasome (Figure 3, Panel B). At this time, sensitivity is optimal. Temperature fluctuations will affect the luminescent readings; if the room temperature fluctuates too much, a constant-temperature incubator may be desired.
3. Record luminescence with a plate-reading luminometer.

5. General Considerations

Sensitivity

The bioluminescent Proteasome-Glo™ Assays are more sensitive than comparable fluorescent assays for several reasons. Biological samples can contain naturally fluorescent compounds that contribute to background; the luminescent assay eliminates background contributions from such compounds. The luminescent substrates are not substrates for luciferase until they are cleaved; hence, there is insignificant inherent background. Fluorescence substrates generally depend on a shift in the excitation/emission wavelengths after cleavage by the protease; consequently, there may be some overlap in the emission spectra of the substrate before and after cleavage, creating substantial inherent background. Pre-incubation of the luminescent proteasome substrates with the Luciferin Detection Reagent insures that any contaminating free aminoluciferin is consumed before beginning the assay (Figure 7). Any contaminating free fluorophore remains in a fluorescent assay, contributing to background. The low background also results in a broad linear range for the luminescent assay (4 logs of proteasome concentration; Figures 2 and 4). The assay sensitivity allows the researcher to use less proteasome if screening for inhibitors. We recommend defining the linear range for the particular proteasome preparation.

These coupled-enzyme assays are not dependent on accumulation of cleaved product because the light output is a result of luciferase consuming the aminoluciferin substrate as it is produced by the protease. Maximum sensitivity is achieved as soon as the proteasome and luciferase activities reach a steady-state. Typically this occurs in 10–30 minutes; therefore the assay is extremely sensitive in a short time frame.

Note: Due to the sensitivity of the Proteasome-Glo™ Assays, 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 reporter gene assays.

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.

After rapidly reaching peak luminescence, the signal is relatively stable for several hours (Figure 3). Ultimate signal stability may vary depending on the proteasome preparation used.

Chemicals

The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for various chemical compounds may interfere with 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 up to 1% in the assay and found to have a minimal effect on light output.

5. General Considerations (continued)

Detergents

The Ultra-Glo™ Recombinant Luciferase is generally tolerant of a wide variety of detergents. An exception is SDS, which will destroy the activity. Although a low percentage of SDS is frequently used to assay the chymotrypsin-like activity of the proteasome, it is not necessary for activation in this luminescent assay and is detrimental to the luciferase.

6. References

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7. Chauhan, D. *et al.* (2005) A novel orally active proteasome inhibitor induces apopotisis in multiple myeloma cells with mechanisms distinct from Bortezomib. *Cancer Cell* **8**, 407–19.
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7. Related Products

Protease Assays

Product	Size	Cat.#
Proteasome-Glo™ Cell-Based Assay	10ml	G8660
Calpain-Glo™ Protease Assay	10ml	G8501
DPPIV-Glo™ Protease Assay	10ml	G8350

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
DeadEnd™ Colorimetric TUNEL System	40 reactions	G7130

Additional Sizes Available.

Cell Viability and Cytotoxicity Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)	10ml*	G7570
CellTiter-Blue® Cell Viability Assay (Resazurin)	20ml*	G8080
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
CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT)	1,000 assays*	G4000
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml*	G9200
CytoTox-Fluor™ Cytotoxicity Assay	10ml*	G9260
CytoTox 96® Non-Radioactive Cytotoxicity Assay (LDH)	1,000 assays	G1780
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH)	1,000–4,000 assays*	G7891

*Additional Sizes Available.

ADME Assays

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays*	V1401
P450-Glo™ CYP1A1 Assay	50ml*	V8752
P450-Glo™ CYP1B1 Assay	50ml*	V8762
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601

*Additional Sizes Available.

7. Related Products (continued)

Luminometers

Product	Size	Cat.#
GloMax® 96 Microplate Luminometer	1 each	E6501
GloMax® 96 Microplate Luminometer with Single Reagent Injector	1 each	E6511
GloMax® 96 Microplate Luminometer with Dual Reagent Injectors	1 each	E6521
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

8. Summary of Changes

The following changes were made to the 1/16 revision of this document:

1. Patent and disclaimer statements were updated.
2. The document design was updated.