Measurement of Three Proteasome Proteolytic Activities Using Luminescent Assays

ABSTRACT The Proteasome-Glo™ 3-Substrate System consists of three homogeneous, bioluminescent assays that measure the three proteolytic activities associated with the proteasome. Three substrates have been synthesized to monitor the chymotrypsin-like, trypsin-like and caspase-like activities associated with the proteasome. The Proteasome-Glo™ Assays are designed for use with multiwell plate formats and are ideal for automated high-throughput screening of proteasome activity and inhibition.

By Martha O'Brien, Ph.D.[1], Mike Scurria, B.S.[2], Laurent Bernad, Ph.D[2]., William Daily, Ph.D.[2], James Unch, Ph.D[2]., Kay Rashka, B.S.[1], Sandra Hagen, B.S.[1], Jeri Culp, B.S[1]., Rich Moravec, B.S.[1], Brian McNamara, Ph.D[1]., and Terry Riss, Ph.D[1]., [1]Promega Corporation, [2]Promega Biosciences, Inc.

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INTRODUCTION

In eukaryotic cells, the turnover of intracellular proteins is mediated mainly by the ubiquitin-proteasome pathway, a non-lysosomal proteolytic pathway. The 26S proteasome is a 2.5MDa multiprotein complex found in both the nucleus and cytosol of all eukaryotic cells. It is comprised of a single 20S core particle and 19S regulatory particles at one or both ends (1,2) and 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 them into the 20S core (3–5).

Three major proteolytic activities (chymotrypsin-, trypsin- and caspase-like) are contained within the 20S core linked to discrete sites. Combined, these activities are responsible for much of the protein degradation required for maintenance of cellular homeostasis, including degradation of critical cell-cycle proteins, tumor suppressors, transcription factors, inhibitory proteins and damaged cellular proteins (6,7).

The importance of the proteasome in regulating turnover of proteins involved in cell cycle control, apoptosis, and angiogenesis has led to the recognition of the proteasome as a therapeutic target for cancer (8–11). A first generation proteasome inhibitor, bortezomib (PS-341), is now an approved drug for the treatment of recurring multiple myeloma, and second generation inhibitors are currently being developed (12). The proteasome is also essential for degrading misfolded and aberrant proteins, and impaired proteasome function has been implicated in diseases such as Parkinson's and Alzheimer's (13). Since all three proteolytic activities contribute significantly to protein breakdown and the relative importance can vary widely depending on the protein substrate, monitoring the three activities is essential for understanding the true impact of an inhibitor on protein degradation (14). We have developed three bioluminescent assays to individually monitor the activities of the three proteolytic sites of the proteasome using purified proteasome preparations.

PROTEASOME-GLO™ ASSAY PRINCIPLE

The Proteasome-Glo™ 3-Substrate System[(a,b,c)] (Cat.# G8531, G8532) consists of three homogeneous, bioluminescent assays that measure the three proteolytic activities associated with the proteasome. Peptide-conjugated fluorophores are widely used as substrates for monitoring proteasome activity, but sensitivity of fluorescent assays can be limited for a variety of reasons. We synthesized luminogenic substrates Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnLD-aminoluciferin to monitor the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome, respectively. Each substrate is added to a buffer system optimized for proteasome activity and luciferase activity to make a Proteasome-Glo™ Reagent for the 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 target substrate. Substrate cleavage generates a "glow-type" luminescent signal produced by the luciferase reaction (Figure 1). The Proteasome-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to improve performance across a wide range of assay conditions. The homogeneous Proteasome-Glo™ Assays are designed for use with multiwell plate formats, making the assays ideal for automated high-throughput screening (HTS) of proteasome activity and inhibition.

RAPID, SENSITIVE ASSAYS WITH STABLE SIGNAL

In this coupled-enzyme format, the proteasome and luciferase rapidly reach a steady-state, where the rate of proteasome cleavage of the substrate is equal to the rate of luciferase utilization of the released

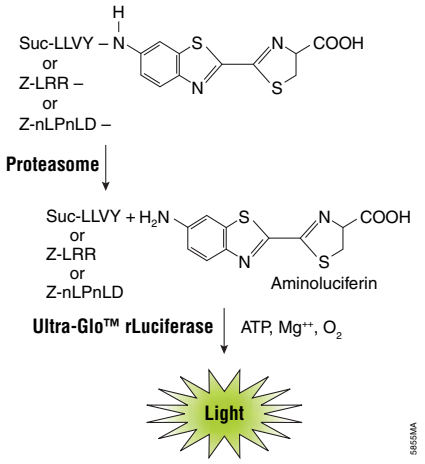

Figure 1. The luminogenic, aminoluciferin substrates containing the Suc-LLVY, Z-LRR, or Z-nLPnLD sequence recognized by the 20S proteasome. Following 20S proteasome cleavage, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to produce light.

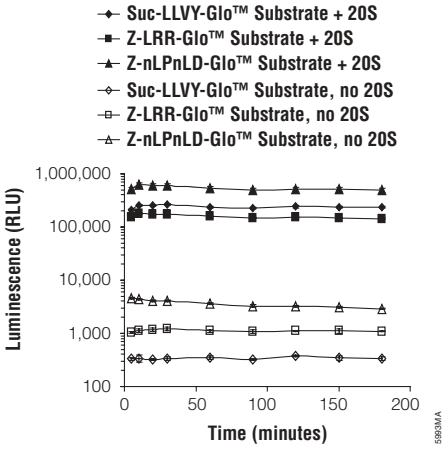

Figure 2. Signal stability of the Proteasome-Glo™ Assays. The Proteasome-Glo™ 3-Substrate System assays were tested with purified human 20S proteasome (1µg/ml; closed symbols) or without 20S proteasome as a control (open symbols) in 96-well plates, 100µl total volume. Luminescence was monitored at various times for 3 hours on a GloMax™ 96 Microplate Luminometer. The signals peak rapidly, then are very stable for all three assays, as shown on a log scale.

aminoluciferin, and stable light output is achieved. Steady-state is typically reached in 10–20 minutes, and stable light output persists for several hours (Figure 2). Eventually the 20S proteasome and the luciferase become inactive and the light output decreases, but the half-life for each of the proteasome assays is greater than three hours (Figure 2).

At steady-state, the light output is proportional to the rate of proteasome cleavage and thus the amount of proteasome activity (Figure 3). Another feature of the homogeneous, bioluminescent format is that any free aminoluciferin that is a by-product of the peptide-conjugating synthesis is removed prior to exposing the proteasome substrate to the test samples. Consequently, the background is very low and the linear dynamic range is

A comparison of bioluminescent and fluorescent proteasome assays demonstrated that the bioluminescent assays are significantly more sensitive and have a much lower limit of detection for proteasome activity (Figure 3).

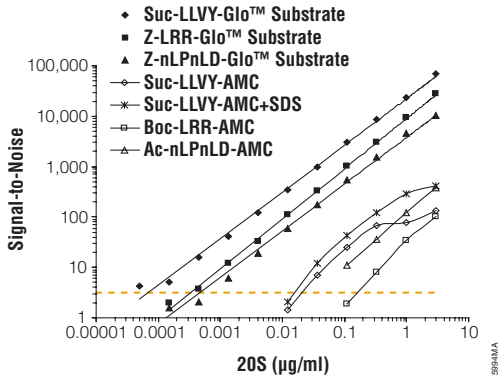

Figure 3. Luminescent proteasome assays are more sensitive than fluorescent proteasome assays. Human 20S proteasome was serially diluted in 10mM HEPES (pH 7.6) in 96-well plates. For each catalytic activity, half of the plate received the appropriate Proteasome-Glo™ Reagent and half of the plate received the comparable fluorogenic substrate, diluted in 100mM HEPES (pH 7.5), 1mM EDTA, to the same concentration as the luminescent substrates. The fluorescent assay for chymotrypsin-like proteasome activity was run with and without 0.02% SDS. Thirty minutes after addition of the Proteasome-Glo™ Reagent, luminescence was recorded as relative light units (RLU) on a GloMax™ 96 Microplate Luminometer. Fluorescence was measured 30 minutes after adding the appropriate substrate on a LabSystems Fluoroskan Ascent fluorometer and recorded as relative fluorescence units (RFU). To normalize between RLU and RFU, the results were plotted as signal-to-noise ratios. Each point represents the average of 4 wells. The Proteasome-Glo™ Assays were linear over 4 logs of 20S proteasome concentration for all three assays. The limit of detection is defined as a signal-to-noise ratio = 3 (horizontal dotted line).

large (Figure 3). The broad linear range and stable signal results in increased sensitivity and flexibility for the bioluminescent proteasome assays. A comparison of bioluminescent and fluorescent proteasome assays demonstrated that the bioluminescent assays are significantly more sensitive and have a much lower limit of detection for proteasome activity (Figure 3). SDS, which is often used to activate the proteasome when measuring the chymotrypsin-like activity, improved the signal-to-noise ratio and linearity of the fluorescent assay using Suc-LLVY-AMC, but the sensitivity still did not approach that of the luminescent assay.

ROBUST, FLEXIBLE AND ACCURATE ASSAYS

The sensitivity and speed of the assays and the flexibility in read time make the Proteasome-Glo™ Assays ideal for high-throughput screening of inhibitors. Since compound libraries are frequently stored in dimethyl sulfoxide (DMSO), we determined the effect of DMSO on the assay. At concentrations up to 1% DMSO, there was no effect on the assay (Figure 4). Between 1 and 10% DMSO, there was a slight decrease in signal for the Chymotrypsin-Like and Caspase-Like Proteasome-Glo™ Assays and a slight increase in the signal for the Trypsin-Like Proteasome-Glo™ Assay. Even at concentrations as high as 10% DMSO, the signal decreased by only 40% for the Chymotrypsin-Like Assay, decreased by only 15% for the Caspase-Like Assay, and increased by 39% for the Trypsin-Like Assay (Figure 4).

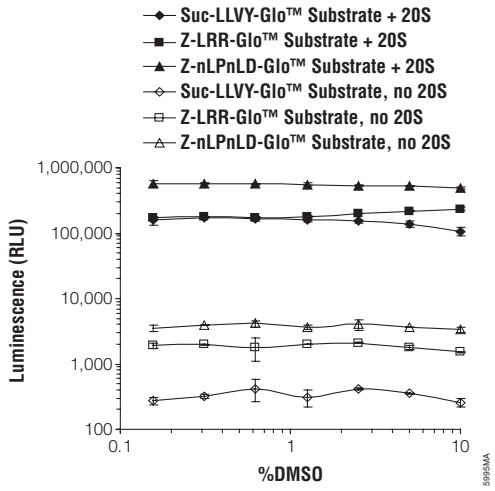

Figure 4. Effect of DMSO on the Proteasome-Glo™ 3-Substrate System. DMSO was titrated in HEPES buffer (10mM, pH 7.6) and combined with human 20S proteasome (Biomol) at 1µg/ml (closed symbols) or control buffer (open symbols) in 96-well plates. The three Proteasome-Glo™ Reagents were each added at 50µl/well for a final volume of 100µl/well. The 20S final concentration was 0.5µg/ml. Luminescence was monitored on a GloMax™ 96 Microplate Luminometer for various times. The 45-minute data is presented in the graph. Each data point is the average of 4 wells.

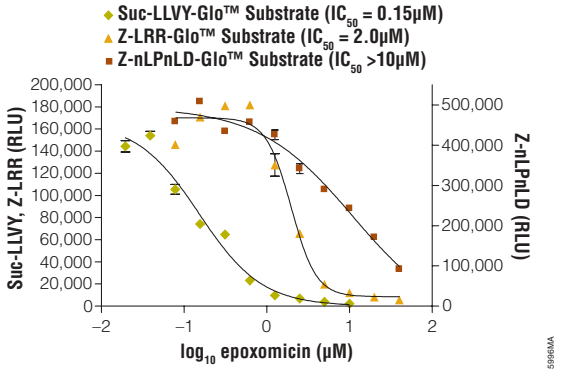

Figure 5. Inhibition of proteasome with epoxomicin. The inhibitor (Biomol) was resuspended in DMSO and diluted in HEPES buffer (10mM, pH 7.6). The highest final DMSO concentration was 0.5%. Human 20S proteasome (Biomol) was added to the inhibitor titration (1µg/ml) and incubated for 20 minutes to allow for irreversible inhibition. The three Proteasome-Glo™ Reagents were each added at 50µl/well for a final volume of 100µl/well. Luminescence was monitored on a GloMax™ 96 Microplate Luminometer for various times. The 60-minute data is presented in the graph. Each point is the average of 4 wells. GraphPad Prism® software was used to calculate the IC_{50} values.

We used the Proteasome-Glo™ 3-Substrate System to test the known, selective proteasome inhibitor, epoxomicin. The epoxomicin inhibits all three catalytic activities irreversibly, with greatest potency for the chymotrypsin-like site, followed by the trypsin-like, and caspase-like sites (Figure 5). The rank order of potency is consistent with previous reports (14,15). The broad linear range of the assay allows for accurate analysis of inhibitors with a broad variety of potencies.

CONCLUSIONS

The Proteasome-Glo™ 3-Substrate System adds three new assays to the expanding list of Promega bioluminescent protease assays. This system includes individual assays for all three catalytic sites of the proteasome and is ideal for high-throughput screening for inhibitors when using 20S or 26S proteasome preparations. These assays complement our novel Proteasome-Glo™ Cell-Based Assay that monitors chymotrypsin-like activity directly in cultured cells. All three assays are significantly more sensitive than their fluorescent counterparts. The sensitivity of the assays allows one to use less purified proteasome and/or less test compound while still achieving accurate results. The assays are readily scaled to a 384-well format, and the stability of the luminescent signal provides great flexibility in read time. The rapid time to maximum sensitivity can make the assays very fast to perform.

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PROTOCOL

- *Proteasome-Glo™ Assay Systems Technical Bulletin* #TB349, Promega Corporation. (www.promega.com/tbs/tb349/tb349.html)

ORDERING INFORMATION

Produce	Size	Cat.#
Proteasome-Glo™ 3-Substrate System	10ml	G8531
	50ml	G8532

[a]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.
[b]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents and patents pending.
[c]Patent Pending.