MONITORING PROTEASOME ACTIVITY WITH A CELL-BASED ASSAY USING A SINGLE-ADDITION LUMINESCENT METHOD

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Here we introduce the Proteasome-Glo™ Cell-Based Assay. This "add-mix-measure" assay measures the chymotrypsin-like protease activity associated with the proteasome in cultured cells.

Introduction

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 capped by 19S regulatory particles at one or both ends (1,2). The barrel-shaped 20S core contains three major proteolytic activities that are classified by the preferred type of amino acid after which they cleave and are routinely described as chymotrypsin-like, trypsin-like and caspase-like or post-glutamyl peptide hydrolyzing. The ubiquitin-proteasome pathway constitutes a major, nonlysosomal means to maintain cellular homeostasis by degrading misfolded or unassembled proteins and contributes to the activation or suppression of many short-lived critical cell-cycle proteins, tumor suppressors, transcription factors and inhibitory proteins (1,3,4). Here we describe the Proteasome-Glo™ Cell-Based Assay[(a,b)], a homogeneous luminescent assay that measures the chymotrypsin-like protease activity associated with the proteasome complex in cultured cells.

The assay eliminates the initial sample processing tasks and allows measurement of cytosolic proteasome activity directly in a multiwell plate.

Assay Attributes

The Proteasome-Glo™ Cell-Based Assay Reagent contains a luminogenic proteasome substrate, Suc-LLVY-aminoluciferin (succinyl-leucine-leucine-valine-tyrosine-aminoluciferin), within a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. 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). Because of this design, the assay eliminates the initial sample processing tasks (cell harvesting, washing, mechanical extraction, centrifugation and protein determination) associated with fluorescent substrate protocols and allows measurement of cytosolic proteasome activity directly in a multiwell plate.

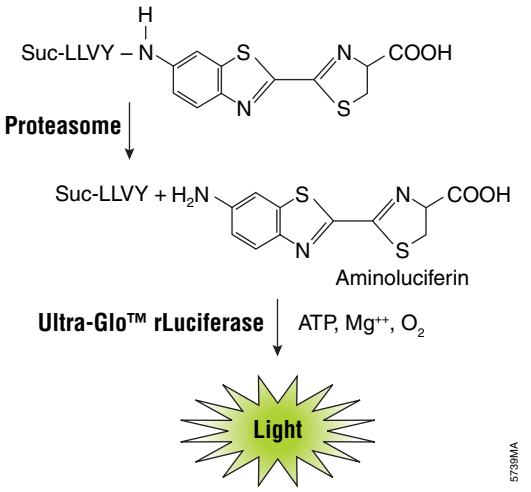

Figure 1. The luminogenic substrate containing the Suc-LLVY sequence recognized by the proteasome. Following proteasome cleavage, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to proceed and produce light.

The Proteasome-Glo™ Cell-Based Assay Reagent contains a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase[(b)]) and is formulated to generate a stable, "glow-type" luminescent signal and improve 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 2). A steady state of the proteasome and luciferase enzyme activities, and hence maximum sensitivity, is reached within 10–15 minutes after adding the reagent, allowing fast and easy monitoring of activity (Figure 3). Glow signal kinetics vary somewhat between cell types, but signal half-lives are generally greater than three hours.

Bioassays Using Cultured Cells

Proteasome activity and the inhibition of that activity are typically monitored using either commercially available purified 20S proteasome or crude cellular extracts. Preparing cell extracts can be laborious and time-consuming and is not conducive to high-throughput screening.

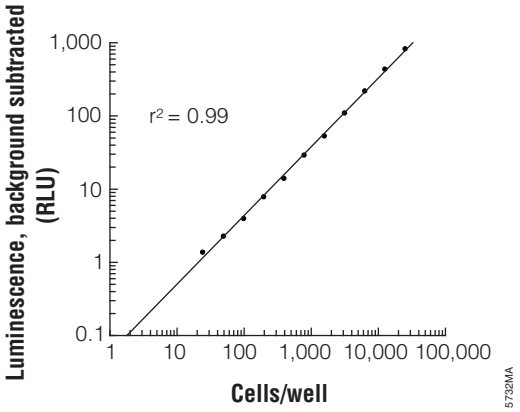

Figure 2. Luminescence is proportional to cell number. A titration of untreated U266 cells was performed in a 96-well plate using the Proteasome-Glo™ Cell-Based Assay. U266 cells (human plasma myeloma) were serially diluted in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate at 100µl/well samples. Cells were then allowed to equilibrate in a humidified 37°C, 5% CO_2 incubator for 1.5 hours. Proteasome-Glo™ Cell-Based Assay Reagent was 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 over three logs of cells (r^2 = 0.99, slope = 0.94). The background (no-cell control) was subtracted from each (average no-cell RLU = 4.135). r^2 value and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot.

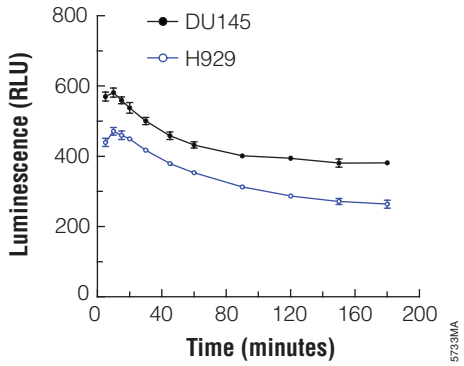

Figure 3. Signal half-life and kinetics of the Proteasome-Glo™ Cell-Based Assay. To demonstrate signal peak and decay, 10,000 cells per well of either DU145 (human prostate line cultured in MEM containing 10% FBS, 1mM sodium pyruvate and 1X nonessential amino acids) or H929 cells (human plasma myeloma cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) were added to a 96-well plate. Cells were then equilibrated in a humidified 37°C, 5% CO_2 incubator for 1.5 hours. Proteasome-Glo™ Cell-Based Reagent was prepared and equilibrated at 22°C for 30 minutes before use, during which time the assay plate was also equilibrated. After the reagent was 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 water bath immediately after each reading. The signal reached its maximum at approximately 10 minutes and slowly decreased over time.

Experiments using cultured cells can give valuable information about permeability, stability, solubility, synergy and potency of a test compound, whereas using purified 20S proteasome preparations for analyzing inhibitors can miss these complex cellular responses. Lactacystin is an example of a highly specific proteasome inhibitor in which cellular glutathione interacts with the active *clasto*-lactacystin-β-lactone form of the drug (5). The Hsp90 inhibitor 17-AAG, a geldanamycin analog, has been shown in vitro to have synergistic effects on bortezomib (PS-341) activity with multiple myeloma cells (6). The Proteasome-Glo™ Cell-Based Assay overcomes the limitations of using either cell extracts or purified proteasome preparations. The sensitivity achieved with our luminogenic protease substrates (7) has enabled the development of a direct "add-mix-measure" assay sensitive enough to measure the chymotrypsin-like proteasome activity directly in cells in multiwell plates.

The Proteasome-Glo™ Cell-Based Assay Reagent gently permeabilizes the plasma membrane and rapidly allows the Suc-LLVY-aminoluciferin substrate access to the cytosol. The reagent is formulated to minimize disruption of lysosomes and subsequent nonspecific cleavage of the luminogenic substrate by other non-proteasome proteases. We have used the Proteasome-Glo™ Cell-Based Assay to obtain EC_{50} values and potency rankings for several inhibitors such as lactacystin and epoxomicin in a variety of cell lines (Figure 4). Furthermore, the assay is easily miniaturized to a 384-well plate to give a Z´-factor value of 0.77 using only 1,500 cells per well (Figure 5), further demonstrating the sensitivity and reproducible performance of this method.

Many proliferating cell types eventually undergo caspase-dependent apoptosis when treated with proteasome inhibitors (8). We were interested to see if we could sequentially multiplex proteasome and caspase measurements on the same sample to look at the kinetics of epoxomicin treatment and induction of caspase-3/7. To do this, we used a modified, concentrated Apo-ONE® Caspase-3/7 Reagent, which contains the profluorescent caspase substrate $[Z\text{-DEVD}]_2$-Rhodamine 110. As shown in Figure 6, a 1.5-hour pretreatment of H929 plasma myeloma cells with epoxomicin inhibited proteasome activity in a dose-dependent fashion but did not induce caspase-3/7. However, increasing the treatment duration to 4.5 hours slightly lowered the epoxomicin EC_{50} value from 9.1 to 3.1nM (consistent with the drug being an irreversible inhibitor), but more importantly, was long enough to show reliable induction of caspase-3/7 activity as measured by the fluorescent caspase product.

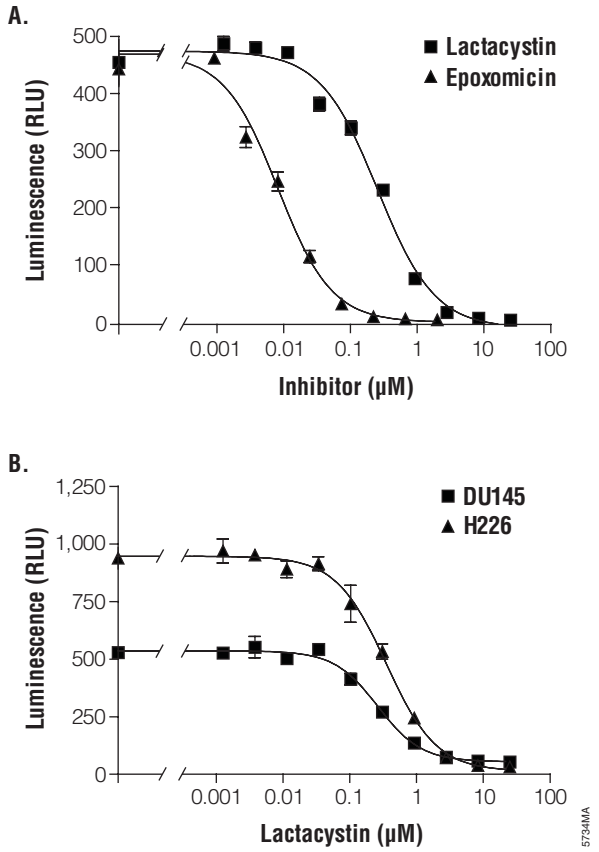

Figure 4. Bioassays using proteasome inhibitors. Panel A. Comparison of lactacystin and epoxomicin using U266 cells. U266 cells (human plasma myeloma; 10,000 cells per well) were plated in 90µl per well volumes in a 96-well plate. Cells were then equilibrated at 37°C, 5% CO_2 for two hours. Serial dilutions 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™ Cell-Based Reagent was added. Luminescence was measured with a DYNEX MLX® luminometer 15 minutes after adding reagent. **Panel B.** Inhibition curves using DU145 and H226 cells. DU145 cells (human prostate; 5,000 cells/well) and H226 (human lung; 2,500 cells per well) were plated in 90µl per well volumes 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 added to the 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™ Cell-Based Reagent was added. Luminescence was measured as described for Panel A.

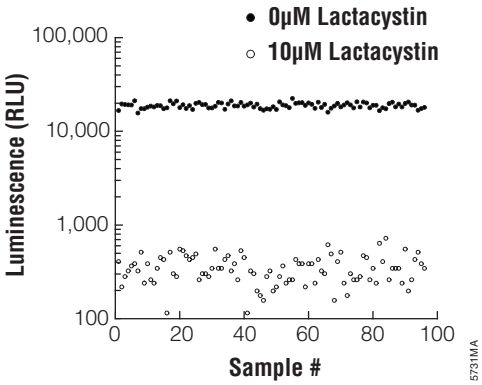

Figure 5. Z´-factor analysis in 384-well format. Z´-factor values for the Proteasome-Glo™ Cell-Based Assay were determined using U266 cells with and without 10µM lactacystin treatment. Cells were dispensed (1,500 cells per 5µl) using a Tecan Freedom EVO® 200 liquid handling system and allowed to equilibrate in a humidified 37ºC, 5% CO_2 incubator for 1.5 hours before lactacystin or vehicle was added in 5µl volumes. Following a 105-minute incubation at 37ºC, 5% CO_2, the assay plate was equilibrated to ambient temperature before adding the Proteasome Glo™ Cell-Based Reagent (10µl/well). Luminescence was determined using a Tecan GENios Pro® luminometer. Ninety-six wells contained untreated cells, and 96 wells in the same plate contained cells treated with 10µM lactacystin. Z´-factor value = 0.77 for this assay. Z´-factor values are statistical indicators 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 (9).

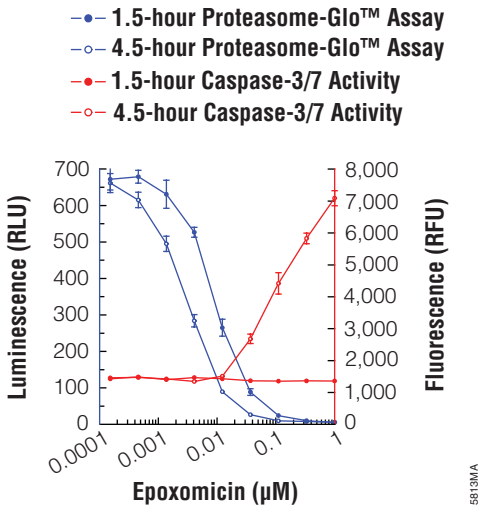

Figure 6. Sequential multiplex to determine proteasome and caspase-3/7 activity. H929 cells (10,000 cells/well) were plated in 90µl per well volumes in a 96-well plate. Following an overnight culture at 37°C, 5% CO_2, cells were incubated with epoxomicin (10µl/well addition) for 1.5 or 4.5 hours at 37°C, 5% CO_2. The plate was removed and allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Cell-Based Assay Reagent was added. Proteasome-mediated luminescence was measured after 15 minutes as described for Figure 2. Immediately, 20µl/well of a modified concentrated Apo-ONE® Homogeneous Caspase-3/7 Reagent, prepared by diluting the Apo-ONE® Substrate 1:20 into the Apo-ONE® Buffer, was added to the samples. The assay plate was mixed by shaking and incubated at 22ºC for an additional 30 minutes before determining caspase-3/7 fluorescence at 485nm$_{Ex}$/527nm$_{Em}$ using a Labsystems Ascent plate fluorometer. GraphPad Prism® was used to obtain EC_{50} values for epoxomicin.

Summary

The Proteasome-Glo™ Cell-Based Assay combines the sensitivity of our luminescent protease assays with the simplicity of a single reagent addition. The homogeneous format of this assay eliminates tedious sample processing. The assay enables cell-based measurement of the chymotrypsin-like activity of the proteasome directly in multiwell plates. The simplified method gives results in just 10–15 minutes with a variety of cell types. An "add-mix-measure" protocol was miniaturized to a 384-well plate format and resulted in a Z′-factor value of 0.77. Finally, we were also able to sequentially multiplex this assay with a modified fluorescent Apo-ONE® Homogeneous Caspase-3/7 Reagent to monitor caspase-3/7 induction following epoxomicin inhibitor treatment. ■

References

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Protocol

Proteasome-Glo™ Cell-Based Assay Technical Bulletin #TB346, Promega Corporation. (www.promega.com/tbs/tb346/tb346.html)

Ordering Information

Product	Size	Cat.#
Proteasome-Glo™ Cell-Based Assay	10ml	G8660
	5 × 10ml	G8661
	2 × 50ml	G8662

For Laboratory Use.

Related Products	Size	Cat.#
Apo-ONE® Homogeneous	1ml	G7792
Caspase-3/7 Assay	10ml	G7790
	100ml	G7791

[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 pending.