



Promega

Technical Manual

DUB-Glo™ Protease Assay (DUB/SENP/NEDP)

INSTRUCTIONS FOR USE OF PRODUCTS G6260 AND G6261.



DUB-Glo™ Protease Assay (DUB/SEN/P/NEDP)

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

1. Description.....	1
2. Product Components and Storage Conditions	8
3. Reagent Preparation and Usage.....	9
4. Protocol for Detection of DUB/SEN/P/NEDP Activity.....	12
A. Assay Conditions.....	12
B. Standard Assay	13
5. General Considerations.....	14
6. References	20
7. Related Products	22

1. Description

The DUB-Glo™ Protease Assay (DUB/SEN/P/NEDP)^(a,b) is a homogeneous, bioluminescent assay that measures the activity of numerous deconjugating enzymes including deubiquitinating (DUB), deSUMOylating (SEN/P) and deneddylating (NEDP) proteases. These proteases reverse the protein modification by ubiquitin and ubiquitin-like proteins (Ubl proteins) and thus are integral components in the complex mechanisms of posttranslational protein regulation in eukaryotes (1-5).

The DUB-Glo™ Protease Assay provides a luminogenic substrate, Z-RLRGG-aminoluciferin, in a reagent optimized for protease and luciferase activity. The RLRGG sequence is the C-terminal pentapeptide of ubiquitin. A single DUB-Glo™ Reagent is added to test protease samples, resulting in cleavage of the substrate and generation of a glow-type luminescent signal produced by luciferase (Figure 1). In this coupled-enzyme format, luminescence is proportional to the amount of DUB, SEN/P or NEDP1 activity present (Figures 2 and 3).

The DUB-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to

1. Description (continued)

generate a stable glow-type luminescent signal and improve performance across a wide range of assay conditions. The protease and luciferase enzyme activities reach a steady state so that the luminescent signal peaks in approximately 30 minutes and is maintained for several hours with a minimal loss of signal (Figure 4). This results in a rapid, sensitive and flexible assay. The assay system may be used with purified enzyme preparations for a variety of deconjugating proteases (Table 1) and is ideal for automated high-throughput screening of inhibitors (Figures 5 and 6). This luminescent format significantly improves the sensitivity over comparable peptide-based fluorescent assays. In some cases, the DUB-Glo™ Protease Assay achieves comparable or better sensitivity than the fluorescent full-length substrates (Table 1, Figures 9 and 10), while avoiding some of the inherent limitations of the full-length substrates (see Section 5). At 50nM protease concentration, the DUB-Glo™ Protease Assay detected activity from UCH-L3, UCH-L1, Isopeptidase T, USP2, USP8, USP15, yeast Otu1, SARS CoV PLpro, BAP1, SENP1, SENP2, SENP6, SENP7 and NEDP1, but did not detect activity from USP7, USP14, Ataxin-3, A20 or SENP5 (see Table 1, Section 5).

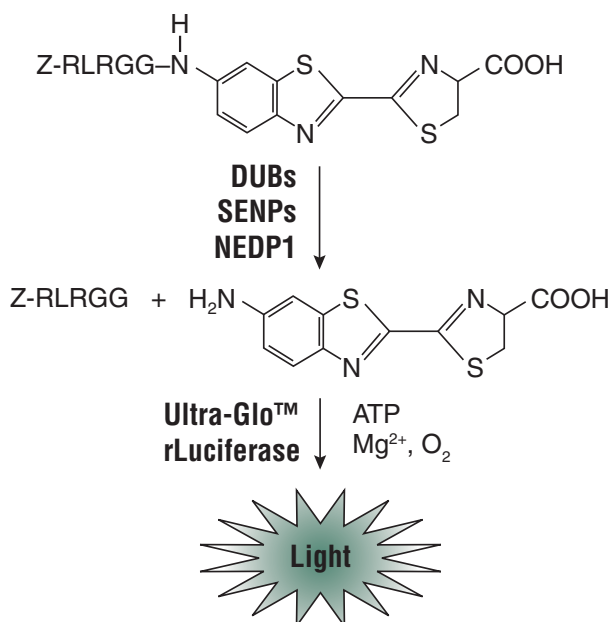


Figure 1. The luminogenic substrate, Z-RLRGG-aminoluciferin, is recognized by numerous deconjugating proteases including DUBs, SENPs and NEDP1.

Following cleavage by the protease, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to produce light.

Assay Advantages

Greater Sensitivity: The luminescent format provides enough sensitivity to enable use of a simple peptide-based substrate, Z-RLRGG-aminoluciferin, for assaying deconjugating proteases. Fluorescence generally requires the use of a full-length conjugated substrate, such as Ub-AMC, SUMO-AMC or Nedd8-AMC (Table 1). The coupled-enzyme format of the DUB-Glo™ Assay results in low background and excellent signal-to-noise ratios (Figure 2).

Broad Dynamic Range: The assays are linear over 2–3 logs of deconjugating protease concentrations (Figure 3). This linearity is maintained for extended time periods due to the stable signal (Figure 4).

Signal Stability: The coupled-enzyme format results in very stable signal with a half-life > 3 hours (Figure 4). Substrate depletion is not a concern as it is when using the full-length substrates, Ub-AMC, SUMO-AMC, or Nedd8-AMC.

Fast: Maximum sensitivity is reached in 10–30 minutes after reagent addition (Figure 4) because the signal is not dependent on accumulation of cleaved product for sensitivity in the coupled-enzyme format.

Accurate and Robust: The broad linear range and excellent sensitivity readily translate to accurate kinetic analysis of inhibitors (Figure 5). Assays can be scaled to 384-well with suitable Z' factors (Figure 6).

Greater Flexibility: The K_m values for the peptide substrates are much higher than they are for the full-length substrates, yet the sensitivity of the luminescent assay allows the assay to be run significantly below K_m while still achieving good signal-to-background ratios for extended time periods. A single luminescent substrate concentration can be used for a wide variety of DUB/SEN/P/NEDP proteases without worrying about substrate depletion or substrate inhibition.

Batch-Processing Capability: The homogeneous coupled-enzyme format results in a continuous signal, providing excellent stability and allowing plates to be read over an extended period of time (Figure 4). Luminometers with reagent injectors are not required.

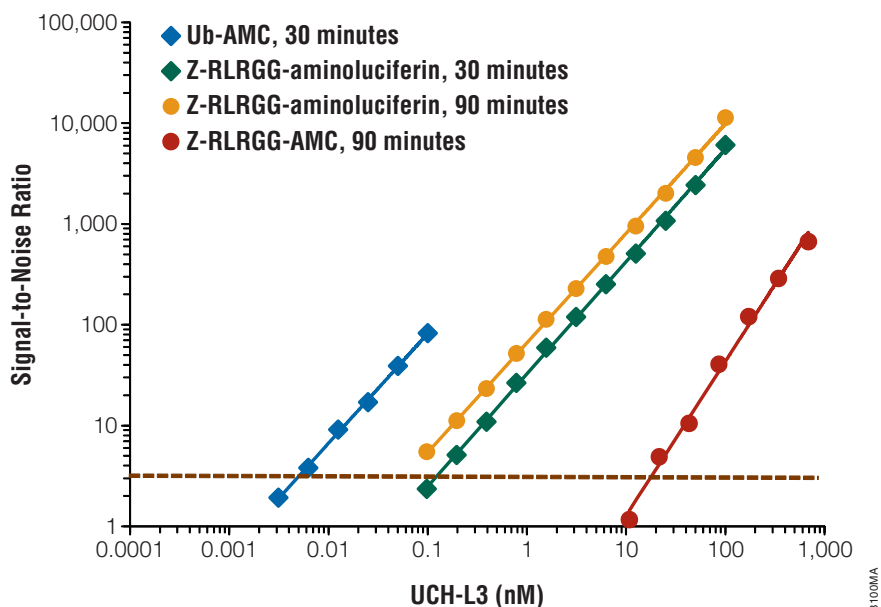


Figure 2. A comparison of the DUB-Glo™ Protease Assay to fluorescent assays for UCH-L3. Human recombinant UCH-L3 was titrated in 50mM HEPES (pH 7.2), 10mM DTT, 0.1mM EDTA and 0.1% Prionex® and assayed in 96-well plates using the DUB-Glo™ Protease Assay, Ubiquitin-AMC (250nM) or Z-RLRGG-AMC (40µM). The fluorogenic substrates were diluted in 50mM HEPES (pH 7.8), 10mM DTT, 0.1mM EDTA and 0.1% Prionex®. Luminescence and fluorescence were measured at various times on a GloMax® 96 Microplate Luminometer (Cat.# E6501) or a Labsystems Fluoroskan Ascent plate reader, respectively. The results are plotted as signal-to-noise ratios. The limit of detection is the amount of UCH-L3 giving a signal-to-noise ratio >3 (dashed line). The sensitivity of the DUB-Glo™ Protease Assay is intermediate between Ub-AMC (more sensitive) and Z-RLRGG-AMC (less sensitive). Additional comparisons are listed in Table 1.

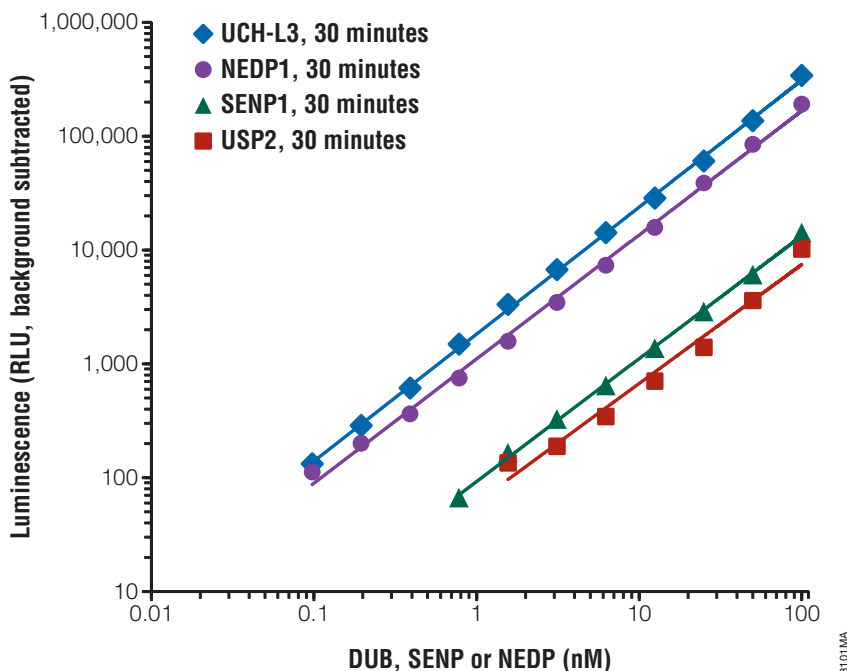


Figure 3. Titration of DUB/SENP/NEDP enzymes assayed in 96-well plates using the DUB-Glo™ Protease Assay. Recombinant human proteases UCH-L3, NEDP1, SENP1 (Boston Biochem) and a rat recombinant USP2 (Enzo Life Sciences) were serially diluted in 50mM HEPES (pH 7.2), 10mM DTT, 0.1mM EDTA, and 0.1% Prionex®. Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer (Cat.# E6501) 30 minutes after adding the DUB-Glo™ Reagent. The assays are linear over 2–3 logs of DUB/SENP/NEDP concentration ($R^2 = 0.99$, slope = 1.0–1.1). Each point represents the average of 4 wells. The no-protease background value was subtracted from each datapoint. R^2 and slope were calculated after transforming the data to a \log_{10} - \log_{10} plot.

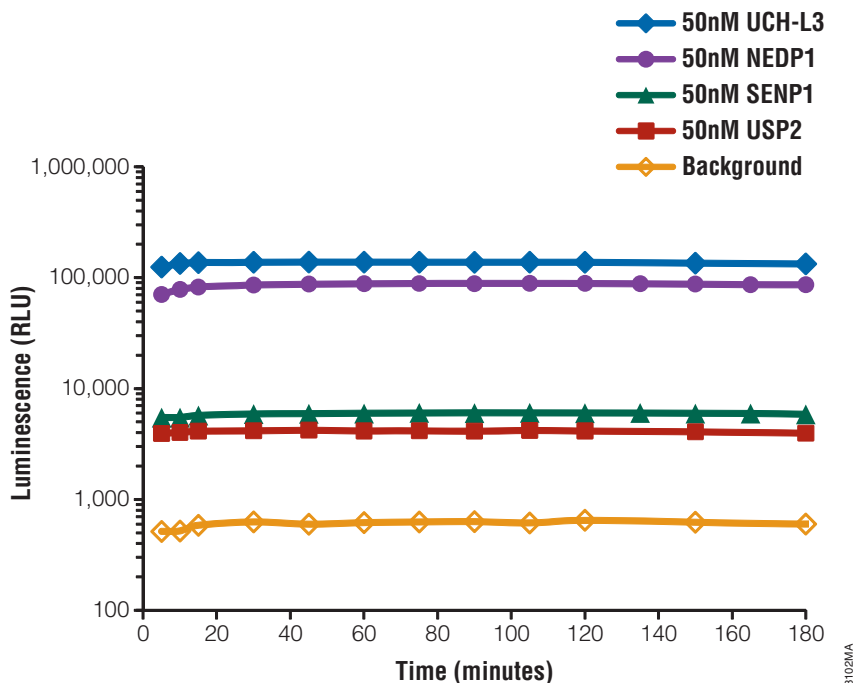


Figure 4. Signal stability of the DUB-Glo™ Protease Assay. Recombinant human proteases, UCH-L3, NEDP1, SENP1 (Boston Biochem) and rat recombinant USP2 (Enzo Life Sciences) were assayed at 50nM in 96-well plates using the DUB-Glo™ Protease Assay. Luminescence was monitored for at least 3 hours on a GloMax® 96 Microplate Luminometer (Cat.# E6501). The assay gives a very stable signal for several hours as shown on a log scale. The half-life for the assay for all the proteases is > 3 hours.

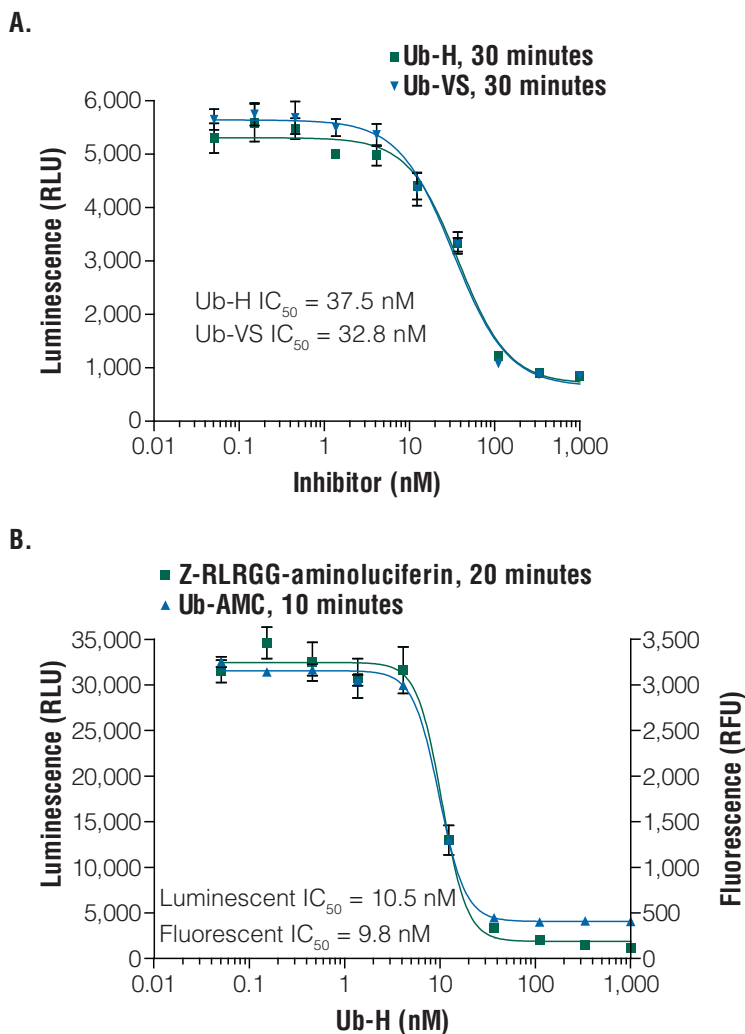
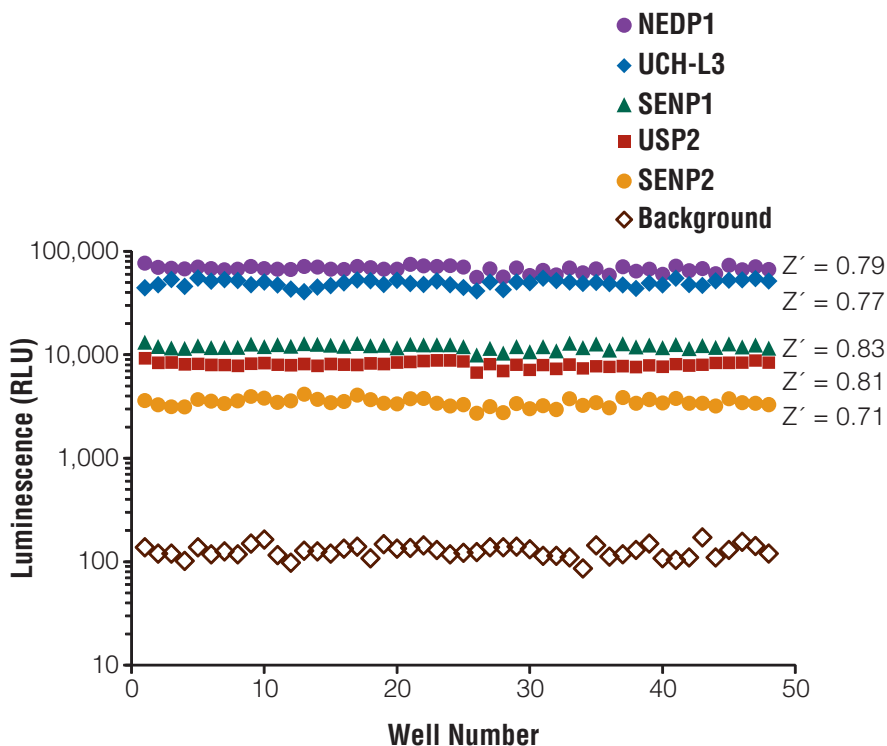


Figure 5. Determination of IC_{50} values for DUB inhibitors. **Panel A.** The inhibitor concentrations that result in 50% inhibition (IC_{50}) of USP2 activity were determined for the ubiquitin-aldehyde (Ub-H) and ubiquitin-vinyl sulfone (Ub-VS) inhibitors using the DUB-Glo™ Protease Assay. Inhibitors were titrated in 50mM HEPES (pH 7.2), 10mM DTT, 0.5mM EDTA and 0.1% Prionex®, combined with rat recombinant USP2 (50nM), and incubated for 60 minutes before adding the DUB-Glo™ Reagent. Luminescence was recorded 30 minutes after reagent addition, and GraphPad Prism® software was used to calculate the IC_{50} . **Panel B.** The IC_{50} for Ub-H inhibition of Isopeptidase T (USP5) was determined using the DUB-Glo™ Protease Assay or Ub-AMC. The Ub-H inhibitor was titrated in 50mM HEPES (pH 7.2), 10mM DTT, 0.5mM EDTA and 0.1% Prionex®, combined with Isopeptidase T (10nM) and ubiquitin (250nM), and incubated for 60 minutes before adding the DUB-Glo™ Reagent or Ub-AMC (500nM final concentration). Luminescence or fluorescence was recorded at the noted times after reagent addition, and GraphPad Prism® software was used to calculate the IC_{50} . The IC_{50} results were the same for both formats, but the dynamic range was much larger for the DUB-Glo™ Protease Assay.



8104MA

Figure 6. Z'-factor analysis in 384-well plates. Z'-factor values (6) for the DUB-Glo™ Protease Assay were calculated using recombinant UCH-L3 (50nM), NEDP1 (200nM), USP2 (200nM), SENP1(400nM) and SENP2 (400nM) and a no-protease control. Recombinant proteases were diluted in 50mM HEPES (pH 7.2), 10mM DTT, 0.5mM EDTA and 0.1% Prionex® as a carrier. Assays were performed in a total volume of 20µl (UCH-L3) or 40µl (other proteases) in a 384-well plate. Luminescence was recorded on a Tecan Safire2™ at 30 minutes.

2. Product Components and Storage Conditions

Product	Size	Cat.#
DUB-Glo™ Protease Assay (DUB/SENP/NEDP)	10ml	G6260

Cat.# G6260 provides sufficient reagents 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:

- 10ml DUB-Glo™ Buffer
- 100µl Z-RLRGG-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent (lyophilized)

Product	Size	Cat.#
DUB-Glo™ Protease Assay (DUB/SENP/NEDP)	50ml	G6261

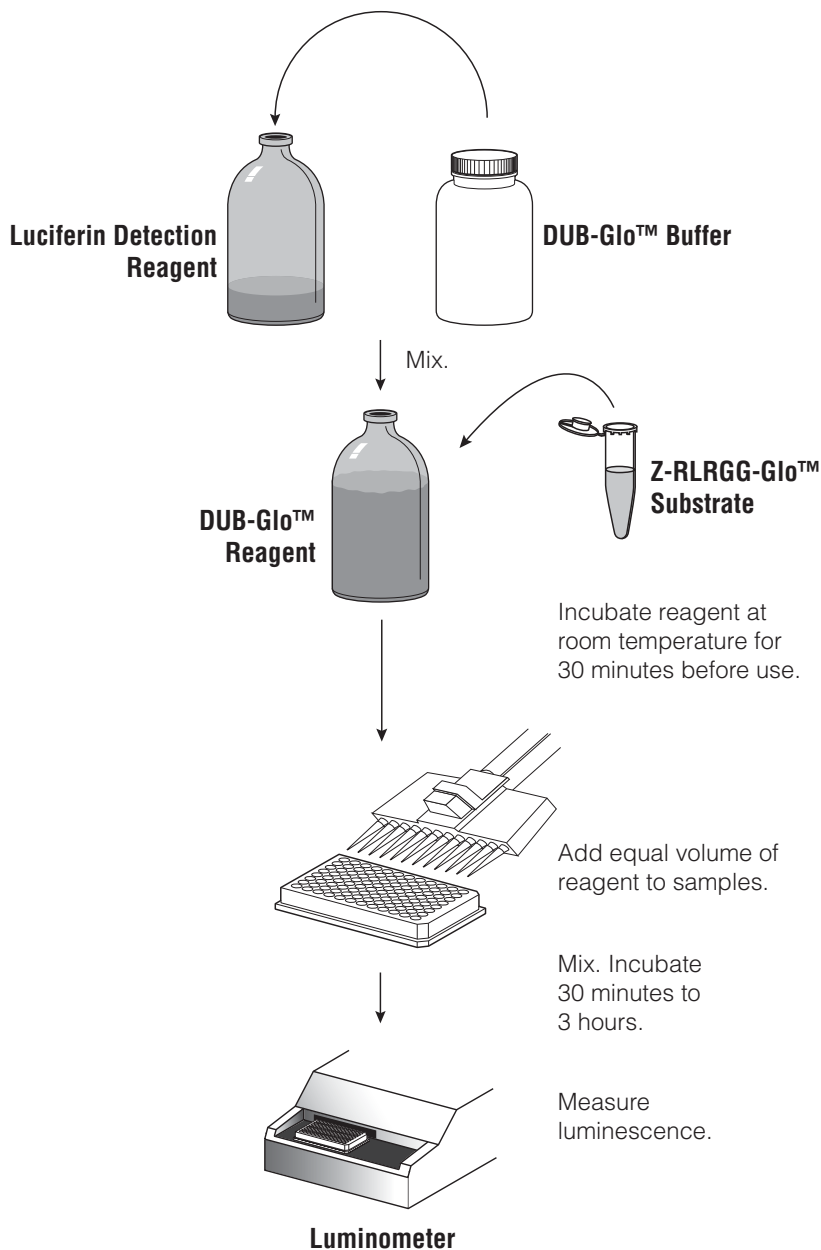
Cat.# G6261 provides sufficient reagents for 500 assays at 100µl/assay or 1,000 assays at 50µl/assay in 96-well plates, or 2,000 assays at 25µl/assay in 384-well plates. Includes:

- 50ml DUB-Glo™ Buffer
- 500µl Z-RLRGG-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent (lyophilized)

Storage Conditions: Store the DUB-Glo™ Protease Assay components at -20°C protected from light. The DUB-Glo™ Buffer may be thawed and stored at 4°C for 2 days or 24 hours at room temperature with no loss in signal. The Z-RLRGG-Glo™ Substrate can be refrozen and stored at -20°C for 1 year with minimal loss of signal.

3. Reagent Preparation and Usage

1. Thaw the DUB-Glo™ Buffer, and equilibrate both the buffer and lyophilized Luciferin Detection Reagent to room temperature (22-25°C) before use.
2. Reconstitute the lyophilized Luciferin Detection Reagent in the amber bottle by adding DUB-Glo™ Buffer (10ml for Cat.# G6260, 50ml for Cat.# G6261). The Luciferin Detection Reagent should go into solution easily in less than one minute.
3. Thaw the Z-RLRGG-Glo™ Substrate, and mix well by vortexing briefly before use.
4. Prepare the DUB-Glo™ Reagent by adding the Z-RLRGG-Glo™ Substrate to the resuspended Luciferin Detection Reagent. For Cat.# G6260, add 100µl of Z-RLRGG-Glo™ Substrate to the 10ml of Luciferin Detection Reagent. For Cat.# G6261, add 500µl of the Z-RLRGG-Glo™ Substrate to the 50ml of Luciferin Detection Reagent. Mix by swirling or inverting the contents to obtain a homogeneous solution. The Z-RLRGG-Glo™ Substrate will be at a 40µM concentration in the DUB-Glo™ Reagent. The DUB-Glo™ Reagent is stable for at least 4 weeks at -20°C. The DUB-Glo™ Reagent can be stored overnight at 4°C or room temperature with minimal loss of signal.
5. Allow the DUB-Glo™ Reagent to sit at room temperature for 30 minutes prior to use. This allows time for the removal of any contaminating free aminoluciferin, ensuring maximal sensitivity. Although free aminoluciferin is not detected by HPLC, it is present in trace amounts (Figure 8).



8105MA

Figure 7. Preparation and use of the DUB-Glo™ Reagent.

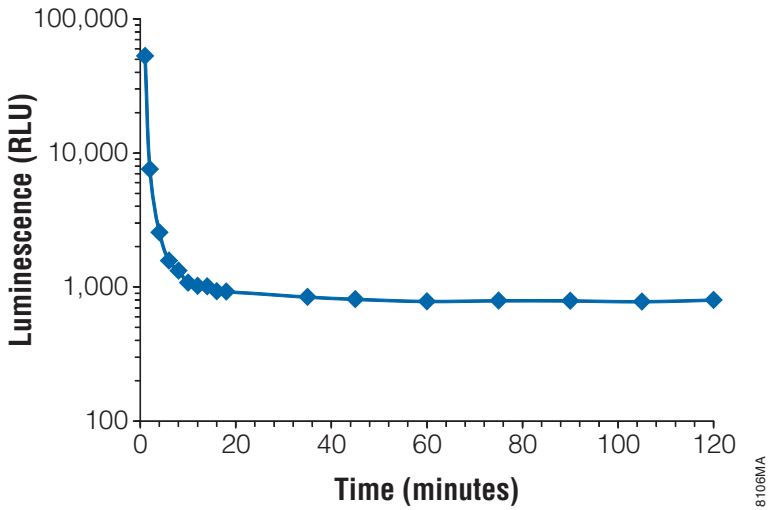


Figure 8. Time course for removal of free aminoluciferin from the DUB-Glo™ Reagent. The Z-RLRGG-Glo™ Substrate was added to the bottle of reconstituted Luciferin Detection Reagent, and a time course of luminescence loss was recorded. Trace amounts of free aminoluciferin are present in the substrate and are removed by incubation with the reconstituted Luciferin Detection Reagent. To achieve maximal assay sensitivity with minimal background luminescence, the prepared DUB-Glo™ Reagent should be incubated for at least 30 minutes at room temperature before use.

4. Protocol for the Detection of DUB/SEN/P/NEDP Activity

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

Materials to be Supplied by the User

- white multiwell plates (if black plates are used, RLU will be reduced)
- multichannel pipette or automated pipetting station for delivery of DUB-Glo™ Reagent
- plate shaker or other device for mixing multiwell plates
- luminometer capable of reading multiwell plates
- UCH-L3 enzyme as positive control [Boston Biochem Cat.# E-325 or Enzo Life Sciences Cat.# UW9745]
- DUB/SEN/P/NEDP enzyme (several available from Boston Biochem or Enzo Life Sciences)
- Buffer for the DUB/SEN/P/NEDP enzymes [We recommend 50mM HEPES (pH 7.2), 10mM DTT, 0.5mM EDTA and 0.1% Prionex® for most proteases. However, SENP1 gives a higher signal-to-background ratio if a slightly higher pH buffer is used (HEPES pH 7.8–8.0).]
- Prionex® Carrier (Centerchem, Inc. Norwalk, CT)

4.A. Assay Conditions

Prepare the following reactions to detect DUB/SEN/P/NEDP activity (or inhibition of activity) in purified enzyme preparations:

Blank: DUB-Glo™ Reagent + protease buffer + vehicle control for test compound or inhibitor (if used).

Positive Control: DUB-Glo™ Reagent + UCH-L3 protease (25–50nM).

Inhibitor Positive Control: DUB-Glo™ Reagent + vehicle control + purified test enzyme.

Test Sample: DUB-Glo™ Reagent + test compound + purified test enzyme.

The blank is used as a measure of background luminescence associated with the test compound vehicle and DUB-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.

Notes:

1. Prepare the DUB-Glo™ Reagent as described in Section 3, and mix thoroughly before beginning the assay. Allow the Reagent to sit at room temperature at least 30 minutes before use to remove any contaminating free aminoluciferin.
2. The final concentration of DUB/SENP/NEDP should be within the linear range of the assay (Figure 3). We recommend testing any DUB/SENP/NEDP enzyme that has not been previously tested at a concentration of 100–500nM.
3. The recommended DUB/SENP/NEDP dilution buffer is 50mM HEPES (pH 7.2), 10mM DTT, 0.5mM EDTA and 0.1% Prionex® carrier (optional as a carrier if low enzyme concentrations are used). For SENP1, a higher pH buffer (pH 7.8–8.0) will give a slightly higher signal-to-background ratio.
Note: The DUB/SENP/NEDP dilution buffer can be stored at –20°C for at least one month.
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 ~30 minutes and will have a half-life of several hours (Figure 4).

4.B. Standard Assay

1. Add 50µl of DUB-Glo™ Reagent to each well of a white 96-well plate containing 50µl of blank, control or test sample.

Note: 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 4).

Note: Maximal signal is reached typically within ~30 minutes using purified DUB/SENP/NEDP (Figure 4). 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

Ubiquitin modifies many proteins (more than 1,000 in yeast) and there are at least 9 Ubl proteins (7). An intricate enzymatic system catalyzes the ubiquitin modification of substrate proteins; attachment requires the consecutive action of three enzymes. These modifications are reversible and are carried out by deconjugating proteases. Deubiquitinating (or deubiquitylating) proteases are termed DUBs and deSUMOylating proteases are termed SENPs for sentrin-specific proteases. Sentrins are an alternative name for SUMOs (small ubiquitin-like modifiers 1, 2 and 3). These two families of proteases are unrelated but have analogous functions: 1) they cleave the C-terminal extensions of immature ubiquitin or SUMO to expose the C-terminal diglycine of the mature protein (a standard peptidase function) and; 2) they hydrolyze the isopeptide linkage to cleave ubiquitin or SUMOs from conjugated substrates or polymeric chains (an isopeptidase function). The term DUB or SENP is generally applied to hydrolases involved in either or both functions (1-5,8,9). The human Nedd8-specific protease (NEDP1), also named deneddylase1 (DEN1), is a member of the SENP family and was initially named SENP8 (10,11). Subsequently, the human NEDP1 was shown specifically to process the Nedd8 precursor and to deconjugate Nedd8 from cullin proteins, important components of the ubiquitin ligase complex. *Drosophila* mutant studies suggest that NEDP1 may also function in deconjugating Nedd8 from non-cullin proteins (12).

Most DUBs and other Ubl proteases (Uls) are cysteine proteases. Labeled peptide substrates are standard substrates for other cysteine and serine proteases, but for the deconjugating proteases, numerous studies have demonstrated that short fluorogenic peptide substrates are very poor substrates relative to fluorogenic substrates incorporating the full-length ubiquitin or Ubl protein (13-19). In most cases the fluorogenic peptide substrates are not processed efficiently enough to detect any activity (14-16, Table 1). The improved sensitivity of luminescence overcomes this obstacle (20-22) and allows the use of a peptide substrate for detecting a variety of DUBs, SENPs, and NEDP1 in vitro. Nedd8 ends in LRGG, and although the SUMOs end in QTGG (23), it has been shown with combinatorial peptide library analysis that two of the six mammalian SENPs, SENP6 and 7, prefer the LRGG sequence over QTGG (24,25). SENP1 and SENP2 also cleave the Z-RLRGG-aminoluciferin substrate efficiently. In addition, recent evidence has demonstrated that viral proteases have DUB and deISGylating functions (26-29). The viral protease, SARS Coronavirus PLpro, readily cleaves the Z-RLRGG-aminoluciferin substrate (Table 1, A. Mesecar, unpublished results).

Table 1. Summary of the comparisons of the DUB-Glo™ Protease Assay to fluorescent assays. The DUB-Glo™ Protease Assay was tested with a variety of DUB, SENP and NEDP1 proteases and was compared to the fluorescent substrates, including the peptide-based substrates and the appropriate full-length ubiquitin or Ubl-conjugated substrate. Qualitative comparisons were made based on signal-to-noise ratios and limits of detection for a titration of each protease.

DUB	Z-RLRGG-Aminoluciferin	Z-RLRGG-AMC or Z-LRGG-AMC	Ub-AMC
UCH-L3	++++	+	+++++
UCH-L1	+	-	+++
Isopeptidase T	+++	+	+++
USP2	++	-	+++
USP8	+++	-	++
USP15	++	-	++
yeast Otu1	++++	+	++++
SARS CoV PL _{pro}	+++	NT	NT
BAP1	+	-	++
USP7	-	-	++
Ataxin-3	-	-	+
USP14	-	-	-
A20	-	-	-
SENP	Z-RLRGG-Aminoluciferin	Z-RLRGG-AMC or Z-LRGG-AMC	SUMO1-AMC
SENP1	+++	-	++++
SENP2	++	-	++
SENP5	-	-	NT
SENP6	+++	-	++
SENP7	+++	-	++
NEDP	Z-RLRGG-Aminoluciferin	Z-RLRGG-AMC or Z-LRGG-AMC	NEDD8-AMC
NEDP1	+++	-	++

(-) No signal above background was achieved with 100nM protease in 2 hours.

NT = Not tested.

Limits of Detection:

+++++ = <0.01nM

++++ = 0.01-0.1nM

+++ = 0.1-1nM

++ = 1-10nM

+ = 10-100nM

Yeast Otu1 and BAP1 were a kind gift from Keith Wilkinson, Emory University.

SARS CoV PL_{pro} was tested by Andy Mesecar's Laboratory, University of Illinois at Chicago.

SENP5, 6, and 7 were a kind gift of Guy Salvesen and Marcin Drag, Burnham Institute.

5. General Considerations (continued)

Will the DUB-Glo™ Protease Assay work for my deconjugating protease?

There are reported to be 95 putative human DUBs, as well as 6 SENPs, and numerous proteases specific for other Ubl proteins. DUBs can be divided into five subfamilies: ubiquitin-specific proteases (Usp or UBP), ubiquitin carboxy-terminal hydrolases (UCH), ovarian tumor-like proteases (OTU), the Ataxin-3/Josephin domains (also called the Machado-Jakob-disease proteases-MJD) and the JAMM/MPN metalloproteases (2). With the exception of the JAMMs that require zinc to catalyze the reaction, all other families use an active site cysteine. Table 1 provides information on the testing of a variety of deconjugating proteases from different subfamilies, but this is a small subset of the total number. Many DUBs bind ubiquitin with high or very high affinities, but there are also DUBs that have little affinity for ubiquitin, but exhibit robust catalytic capability. Presumably there are other requirements for binding substrate such as the presence of the target protein or macromolecular complexes (2,30). In those cases, neither the full-length substrate nor peptide-based substrates alone will be suitable. Likewise, if a particular deconjugating protease is restricted to isopeptidase activity, neither the full-length substrates nor peptide-based substrates would be effective. In a small number of cases, there may be an absolute requirement for the full-length substrate. For instance, USP7 efficiently cleaved Ub-AMC but had no detectable activity with the Z-RLRGG-aminoluciferin substrate. If the protease of interest is not listed, we recommend that the DUB-Glo™ Protease Assay be tested with the unknown protease at 100–500nM, using UCH-L3 (50nM) as a positive control. In general, if Ub-AMC generates a strong signal, it is anticipated that the DUB-Glo™ Protease Assay will give adequate signal in most cases. If no signal is detected with 100–500nM protease, it is expected that there are other requirements for substrate specificity (2,30,31).

Is the substrate concentration in the DUB-Glo™ Protease Assay suitable for my protease? Some DUBs and SENPs bind ubiquitin or SUMO with very high affinities, so the K_m values for the full-length substrates are extremely low, in the nanomolar range (9,14–17). Using these substrates such as Ubiquitin-AMC or SUMO-AMC at K_m results in rapid substrate depletion limiting the time of the assay window and the dynamic range of the assay (17, Figure 9). The Z-RLRGG-aminoluciferin substrate binds the DUBs and SENPs with much lower affinities, but the higher K_m values give the assay greater flexibility. The substrate concentration in the DUB-Glo™ Protease Assay is well below the K_m for most of the proteases, but the concentration is high enough to give strong signal for a broad dynamic range, without the risk of substrate depletion. The concentration is low enough to give accurate inhibition profiles for reversible competitive inhibitors (Figure 5, Panel B). Using the substrate at the recommended concentration will be appropriate for most deconjugating

proteases. However, the Z-RLRGG-aminoluciferin concentration can be adjusted if desired. The half-life of the DUB-Glo™ Protease Assay is generally greater than 4–5 hours, whereas the half-life for the assays using the full-length substrates typically is in minutes (Figure 9). Another limitation of assays using the full-length substrates is the risk of substrate inhibition of the proteases. Some DUBs and SENPs are known to exhibit substrate inhibition, and this can occur at relatively low concentrations (14, Figure 10). For those deconjugating proteases prone to this phenomenon, full-length substrates are problematic. After the substrate is cleaved, the released full-length protein may reach a concentration that begins to inhibit the protease, complicating the kinetics of the assay. The relatively low IC₅₀ for Sumo-1 inhibition of SENP1 may contribute to the limited range and short half-life of the fluorescent assay (Figures 9 and 10). While substrate depletion and inhibition can be problematic for assays that use full-length substrates, substrate depletion and inhibition do not affect the DUB-Glo™ Protease Assay.

Assay Linearity and Stability. The DUB-Glo™ Protease Assay has a broad linear range, and the linearity is maintained over extended periods (Figures 3, 4 and 9). An inherent property of the DUB or other Ulp that may affect the linearity is dimerization. There is evidence that some DUBs such as USP8 and UCH-L1 may dimerize under certain conditions (15,32); the linear range of the assay may be limited in those situations. The DUB-Glo™ Protease Assay results in a very stable signal that is dependent on the continued activity of the protease. Diluting the protease of interest in buffer containing fresh DTT or DTT from frozen stocks is recommended to maintain maximum activity of these cysteine proteases. DTT stocks that have been stored at 4°C are not suitable. DTT is included in the DUB-Glo™ Buffer, but it is also recommended to dilute the enzyme in buffer containing DTT to maximize the signal stability.

5. General Considerations (continued)

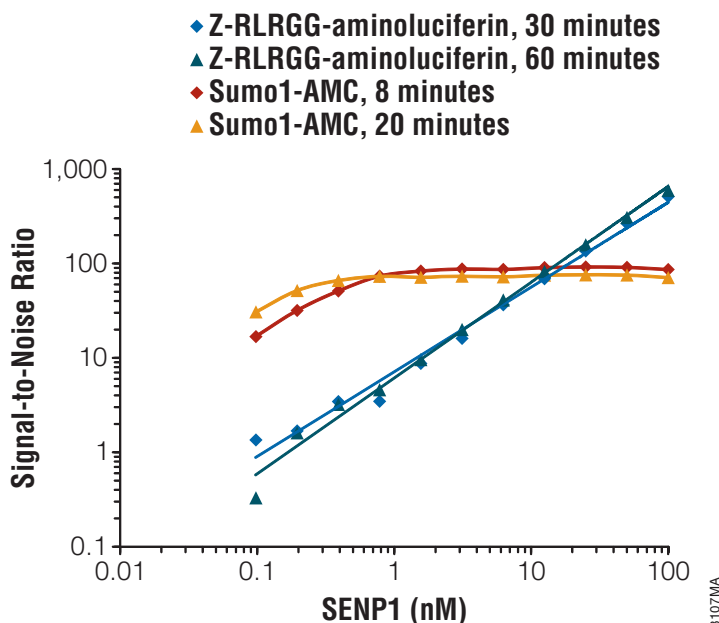


Figure 9. A comparison of the DUB-Glo™ Protease Assay to a fluorescent assay for SENP1. Human recombinant SENP1 (Boston Biochem, Cat. #E-700) was titrated in 50mM HEPES (pH 7.8), 4mM DTT, 0.1mM EDTA and 0.1% Prionex® and assayed in 96-well plates using the DUB-Glo™ Protease Assay or Sumo1-AMC (250nM) diluted in the same buffer (above). Luminescence and fluorescence were measured at various times on a GloMax® 96 Microplate Luminometer (Cat.# E6501) or a Labsystems Fluoroskan Ascent plate reader, respectively. The results are plotted as signal-to-noise ratios. The DUB-Glo™ Protease Assay maintains linearity for extended periods over a broad dynamic range. The fluorescent assay reveals substrate depletion within 8 minutes using 1nM or more SENP1. NEDP1 also demonstrates substrate depletion very rapidly with NEDD8-AMC (data not shown).

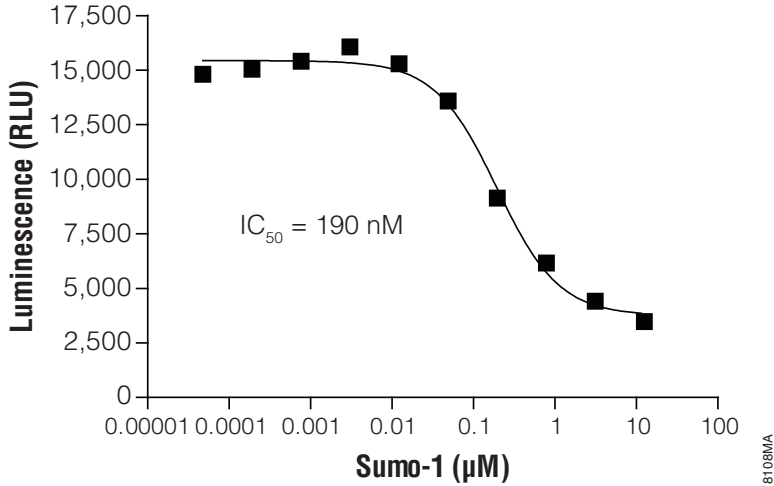


Figure 10. Sumo-1 inhibition of SENP1. Sumo-1 was titrated in 50mM HEPES (pH 7.8), 4mM DTT, 0.1mM EDTA and 0.1% Prionex®, combined with SENP1 (100nM) and incubated for 5 minutes in 96-well plates before assaying with the DUB-Glo™ Protease Assay. Luminescence was measured 30 minutes after reagent addition with a GloMax® 96 Microplate Luminometer (Cat.# E6501). GraphPad Prism® software was used to calculate the IC₅₀. Substrate inhibition is pronounced for SENP1 with Sumo-1, giving an IC₅₀ of 190nM. NEDP1 also demonstrated pronounced substrate inhibition with NEDD8, inhibiting NEDP1 with an IC₅₀ of 85nM (data not shown).

6. References

1. Wilkinson, K.D. (1997) Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J.* **11**, 1245-56.
2. Ventii, K.H. and Wilkinson, K.D. (2008) Protein partners of deubiquitinating enzymes. *Biochem. J.* **414**, 161-75.
3. Hay, R.T. (2007) SUMO-specific proteases: a twist in the tail. *Trends in Cell Biol.* **17**, 370-6.
4. Ponder, E.L. and Bogyo, M. (2007) Ubiquitin-like modifiers and their deconjugating enzymes in medically important parasitic protozoa. *Eukaryotic Cell* **6**, 1943-52.
5. Love, K.R. *et al.* (2007) Mechanisms, biology and inhibitors of deubiquitinating enzymes. *Nature Chem. Biol.* **3**, 697-705.
6. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67-73.
7. Hochstrasser, M. (2009) Origin and function of ubiquitin-like proteins. *Nature* **458**, 422.
8. Lin, H. *et al.* (2001) Divergent N-terminal sequences of a deubiquitinating enzyme modulate substrate specificity. *J. Biol. Chem.* **276**, 20357-63.
9. Mikolajczyk, J. *et al.* (2007) Small ubiquitin-related modifier (SUMO)-specific proteases: Profiling the specificities and activities of human SENPs. *J. Biol. Chem.* **282**, 26217-24.
10. Gan-Erdene, T. *et al.* (2003) Identification and characterization of DEN1, a deneddylase of the ULP family. *J. Biol. Chem.* **278**, 28892-900.
11. Mukhopadhyay, D. and Dasso, M. (2007) Modification in reverse: The SUMO proteases. *Trends in Biochem. Sci.* **32**, 286-95.
12. Chan, Y. *et al.* (2008) DEN1 deneddylates non-cullin proteins in vivo. *J. Cell Science* **121**, 3218-23.
13. Stein, R.L., Chen, Z. and Melandri, F. (1995) Kinetic studies of Isopeptidase T: Modulation of peptidase activity by ubiquitin. *Biochemistry* **34**, 12616-23.
14. Dang, L.C., Melandri, F.D. and Stein, R.L. (1998) Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. *Biochemistry* **37**, 1868-79.
15. Avvakumov, G.V. *et al.* (2006) Amino-terminal dimerization, NRDP1-rhodanese interaction, and inhibited catalytic domain conformation of the ubiquitin-specific protease 8 (USP8). *J. Biol. Chem.* **281**, 38061-70.
16. Renatus, M. *et al.* (2006) Structural basis of ubiquitin recognition by the deubiquitinating protease USP2. *Structure* **14**, 1293-1302.
17. Hassiepen, U. *et al.* (2007) A sensitive fluorescence intensity assay for deubiquitinating proteases using ubiquitin-rhodamine110-glycine as substrate. *Anal. Biochem.* **371**, 201-7.
18. Barretto, N. *et al.* (2005) The papain-like protease of severe acute respiratory syndrome coronavirus has deubiquitinating activity. *J. Virol.* **79**, 15189-98.

19. Drag, M. *et al.* (2008) Positional-scanning fluorogenic substrate libraries reveal unexpected specificity determinants of deubiquitinating enzymes (DUBs). *Biochem. J.* **415**, 367–75.
20. O'Brien, M.A. *et al.* (2005) Homogeneous bioluminescent protease assays: Caspase-3 as a model. *J. Biomol. Screen.* **10**, 137–48.
21. O'Brien, M.A. (2006) A comparison of homogeneous bioluminescent and fluorescent methods for protease assays. In: *Handbook of Assay Development in Drug Discovery*, Taylor and Frances Group (ed. Lisa Minor).
22. Moravec, R.A. *et al.* (2009) Cell-based bioluminescent assays for all three proteasome activities in a homogeneous format. *Anal. Biochem.* **387**, 294–302.
23. Hemelaar, J. *et al.* (2004) Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. *Mol. Cell. Biol.* **24**, 84–95.
24. Drag, M. *et al.* (2008) Activity profiling of human deSUMOylating enzymes (SENPs) with synthetic substrates suggests an unexpected specificity of two newly characterized members of the family. *Biochem. J.* **409**, 461–9.
25. Drag, M. and Salvesen G.S. (2008) DeSUMOylating enzymes – SENPs. *IUBMB Life* **60**, 734–42.
26. Lindner, H.A. *et al.* (2005) The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. *J. Virol.* **79**, 15199–208.
27. Ratia K. *et al.* (2006) Severe acute respiratory syndrome coronavirus papain-like protease: Structure of a viral deubiquitinating enzyme. *Proc. Natl. Acad. Sci. USA* **103**, 5717–22.
28. Chen, Z. *et al.* (2007) Proteolytic processing and deubiquitinating activity of papain-like proteases of human coronavirus NL63. *J. Virol.* **81**, 6007–18.
29. Ratia K. *et al.* (2008) A noncovalent class of papain-like protease/deubiquitinase inhibitors blocks SARS virus replication. *Proc. Natl. Acad. Sci. USA* **105**, 16119–24.
30. Borodovsky, A. *et al.* (2001) A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. *EMBO J.* **20**, 5187–96.
31. Winborn, B.J. *et al.* (2008) The deubiquitinating enzyme Ataxin-3, a polyglutamine disease protein, edits Lys63 linkages in mixed linkage ubiquitin chains. *J. Biol. Chem.* **283**, 26436–43.
32. Das, C. *et al.* (2005) Structural basis for conformational plasticity of the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. *Proc. Natl. Acad. Sci. USA* **103**, 4675–80.

7. Related Products

Product	Size	Cat.#
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay*	10ml	G8660
Proteasome-Glo™ Trypsin-Like Cell-Based Assay*	10ml	G8760
Proteasome-Glo™ Caspase-Like Cell-Based Assay*	10ml	G8860
Proteasome-Glo™ 3-Substrate Cell-Based Assay System*	10ml	G1180
Proteasome-Glo™ Chymotrypsin-Like Assay*	10ml	G8621
Proteasome-Glo™ Trypsin-Like Assay*	10ml	G8631
Proteasome-Glo™ Caspase-Like Assay*	10ml	G8641
Proteasome-Glo™ 3-Substrate System*	10ml	G8531
DPPIV-Glo™ Protease Assay*	10ml	G8350
Calpain-Glo™ Protease Assay*	10ml	G8501
Caspase-Glo® 3/7 Assay*	2.5ml	G8090
Caspase-Glo® 2 Assay*	10ml	G0940
Caspase-Glo® 6 Assay*	10ml	G0970
Caspase-Glo® 8 Assay*	2.5ml	G8200
Caspase-Glo® 9 Assay*	2.5ml	G8210
Protease-Glo™ Assay	each	G9451

*For Laboratory Use. Available in Additional Sizes.

Luminometers

Product	Size	Cat.#
GloMax® 20/20 Luminometer	each	E5311
GloMax® 20/20 Luminometer w/Single Auto-Injector	each	E5321
GloMax® 20/20 Luminometer w/Dual Auto-Injector	each	E5331
GloMax® 96 Microplate Luminometer	each	E6501
GloMax® 96 Microplate Luminometer w/Single Injector	each	E6511
GloMax® 96 Microplate Luminometer w/Dual Injectors	each	E6521
GloMax®-Multi Base Instrument	each	E7031
GloMax®-Multi Luminescence Module	each	E7041
GloMax®-Multi Fluorescence Module	each	E7051
GloMax®-Multi Absorbance Module	each	E7061
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Shaking	each	E8032
GloMax®-Multi+ Detection System with Instinct™ Software: Base Instrument with Heating and Shaking	each	E9032
GloMax®-Multi+ Luminescence Module	each	E8041
GloMax®-Multi+ Fluorescence Module	each	E8051
GloMax®-Multi+ Visible Absorbance Module	each	E8061
GloMax®-Multi+ UV-Visible Absorbance Module	each	E9061

©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.

©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.

© 2009, 2011 Promega Corporation. All Rights Reserved.

Caspase-Glo, GloMax are registered trademarks of Promega Corporation. Calpain-Glo, DPPiV-Glo, DUB-Glo, Instinct, Protease-Glo, Proteasome-Glo, Ultra-Glo and Z-RLRGG-Glo are trademarks of Promega Corporation.

GraphPad Prism is a registered trademark of GraphPad Software, Inc. Prionex is a registered trademark of Pentapharm Ltd. Safire2 is a trademark of Tecan Group, Ltd.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.