Detecting the Deubiquitin Activity of SARS-CoV PLpro: Turning on the Light with the...

Detecting the Deubiquitin Activity of SARS-CoV PLpro: Turning on the Light with the DUB-Glo™ Protease Assay

ABSTRACT

The DUB-Glo™ Protease Assay is a biochemical bioluminescent assay for deubiquitinating proteases and other ubiquitin-like proteases. The severe acute respiratory syndrome coronovirus papain-like protease (SARS-CoV PLpro) has been shown to have deubiquitinating and deisgylating activity and helps enable the virus to evade the human immune system. We demonstrate here how the DUB-Glo™ Protease Assay provides a sensitive means of monitoring SARS-CoV PLpro activity, as well as a variety of other deconjugating proteases.

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Publication Date: 2009

Introduction

Deubiquitinating proteases (DUBs) and ubiquitin-like proteases (Ulps) catalyze the deconjugation of ubiquitin (Ub) and ubiquitin-like (Ubl) proteins from their substrate proteins, respectively, and process the precursors of Ub and Ubl proteins to form mature proteins available for conjugation (Figure 1, Panel A). These proteases are thus integral components in the complex mechanisms of posttranslational protein regulation in eukaryotes. The approximately 100 human DUBs can be divided into five subfamilies: ubiquitin-specific proteases (USP or UBP), ubiquitin carboxy-terminal hydrolases (UCH), ovarian tumor-like proteases (OTU), Machado-Jakob-disease proteases (MJD), and the JAMM/MPN metalloproteases(1) (2) . With the exception of the JAMM metalloproteases, all other families use an active site cysteine. Conjugation of Ub is primarily a signal for targeting proteins to the proteasome for degradation, but Ub also plays diverse roles in the regulation of numerous other biological pathways. There are at least nine Ubl proteins, but the most extensively studied are the SUMOs (small ubiquitin-like modifiers 1, 2, 3), Nedd8, ISG15, and Atg8(3) . These function as critical regulators of transcription, DNA repair, signal transduction, autophagy, cell-cycle control, immune response, and other cellular processes(4) .

Current methods for assaying the activity of the deconjugating proteases have typically required substrates that have the full-length ubiquitin or Ubl protein conjugated with a fluorophore, such as ubiquitin-AMC or Sumo1-AMC. The specificity requirements of the DUBs and Ulps dictate that fluorogenic peptide substrates are generally ineffectual substrates(5) (6) (7) (8) (9) (10) . Here we describe a highly sensitive, homogeneous bioluminescent assay in a coupled-enzyme format, developed to measure deubiquitinating enzyme activity. The improved sensitivity of luminescence has enabled a peptide-based assay for the first time that measures the activity of numerous deconjugating proteases, including DUBs, desumoylating, and deneddylating proteases, and viral proteases exhibiting these activities.

The DUB-Glo™ Protease Assay Principle

The coupled assay uses a luminogenic substrate based on the C-terminal pentapeptide sequence of ubiquitin, Z-RLRGG-aminoluciferin, in a mixture with thermostable firefly luciferase (Ultra-Glo™ Recombinant Luciferase) and its necessary

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cofactors, ATP and magnesium (Figure 1, Panel B). The protease and luciferase enzyme activities reach a steady state so that the luminescent signal peaks in 30-60 minutes and is maintained for several hours with a minimal loss of signal. 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, including several DUBs, four desumoylating or sentrin proteases (SENPs) and the deneddylating protease, NEDP1 (Figure 1, Panel C). Although Sumo has QTGG at its C-terminus, the RLRGG sequence is a better substrate for SENP6 and SENP7(9) and is suitable for SENP1 and SENP2. This luminescent format significantly improves the sensitivity over comparable peptide-based fluorescent assays. Although the K_m of the DUBs and Ulps for peptide substrates are significantly higher than the K_ms for the full-length substrates(11) (5) (6) (7) (8) (9) , the DUB-Glo™ Protease Assay frequently achieves sensitivity comparable to and in some cases, better than the fluorescent full-length substrates(12) . The DUB-Glo™ Protease Assay avoids the problems of substrate depletion and substrate inhibition that are inherent limitations of the full-length substrates (as a result of their very low K_ms). The luminogenic substrate is used in the DUB-Glo™ Protease Assay at a concentration well below K_m, but still gives good signal-to-background ratios and broad dynamic ranges. The assay gives accurate inhibition results as shown for the competitive, reversible inhibitor, ubiquitin-aldehyde and the DUB, isopeptidase T (USP5) (Figure 2). The stability of the signal and flexibility of the DUB-Glo™ Protease Assay make it ideal for automated high-throughput screening of inhibitors.

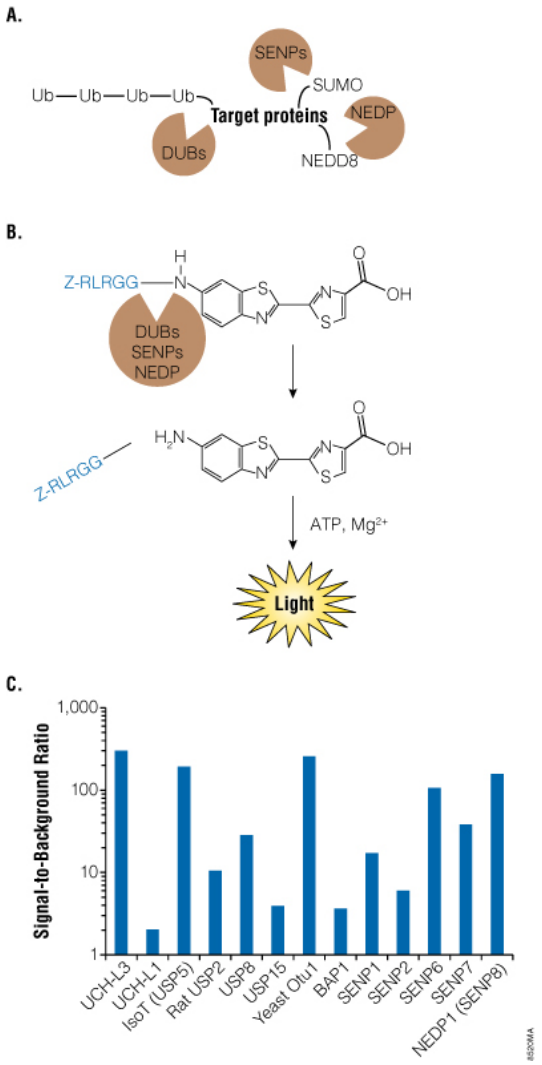


Figure 1. DUB-Glo™ Protease Assay Principle. Panel A. Deubiquitinating proteases (DUBs) and ubiquitin-like proteases (Ulps) catalyze the deconjugation of ubiquitin (Ub) and ubiquitin-like (Ubl) proteins from their substrate proteins, respectively, and process the precursors of Ub and Ubl proteins to form mature proteins available for conjugation. **Panel B.** In the presence of deubiquitinating activity, cleavage of the substrate at the scissile site liberates aminoluciferin, which in turn acts as a luciferase substrate producing luminescent light proportional to protease activity. **Panel C.** DUB-Glo™ Protease Assay exhibits good signal-to-background ratios. A variety of DUB/SENP/NEDPs were tested at a final concentration of 50nM. Recombinant human proteases were used unless otherwise noted. Luminescence was monitored 30 minutes after adding the DUB-Glo™ Reagent on a GloMax® 96 Microplate Luminometer (Cat.# E6501). The coupled-enzyme bioluminescent assay has low background resulting in excellent signal-to-background ratios. UCH-L3, isopeptidase T, yeast Otu1, SENP6 and NEDP1 give high signal-to-background ratios (>100); USP8, USP2, SENP1 and SENP7 give medium ratios (10 to 100); and UCH-L1, USP15, BAP1, and SENP2 give low ratios (<10). The DUBs and SENPs that gave no activity in the DUB-Glo™ assay were USP7, USP14, ataxin-3, A20, and SENP5. Yeast Otu1 and BAP1 were obtained from Keith Wilkinson (Emory University), SENP6 and 7 were obtained from Guy Salvesen and Marcin Drag (The Burnham Institute), and the remaining proteases were purchased from Boston Biochem or Enzo Life Sciences.

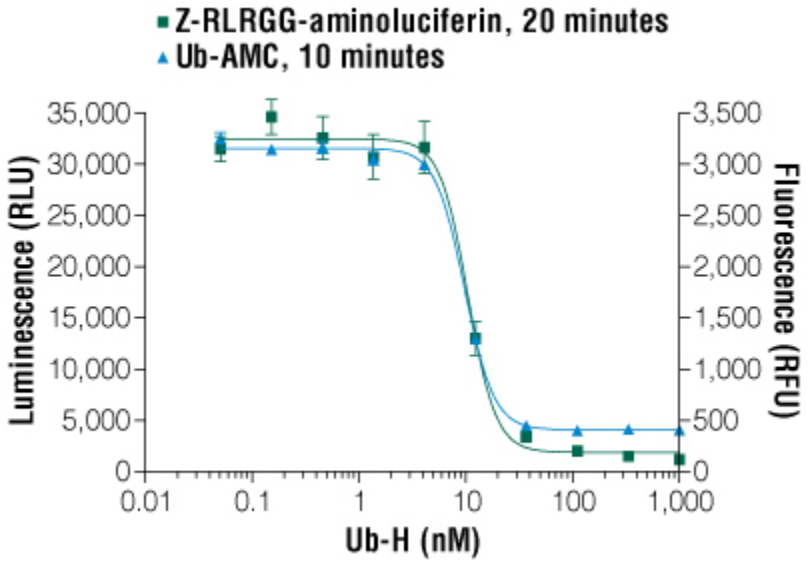


Figure 2. Determining IC_{50} using the DUB-Glo™ Assay. The inhibitor concentrations that result in 50% inhibition (IC_{50}) were determined for ubiquitin-aldehyde (Ub-H) inhibition of Isopeptidase T (USP5) using the DUB-Glo™ Protease Assay or Ub-AMC. The Ub-H inhibitor was titrated in 50mM Hepes (pH 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, on a GloMax® 96 Microplate Luminometer or a Labsystems Fluoroskan Ascent plate reader, respectively. 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.

A Novel and Highly Sensitive Screening Assay for SARS-CoV PLpro

The severe acute respiratory syndrome coronovirus papain-like protease (SARS-CoV PLpro) has been shown to have deubiquitinating and deisgylating activity and helps enable the virus to evade the human immune system(13) (11) (14) (15) (16) . The development of novel antivirals against SARS-CoV, the virus which caused the brief pandemic of 2002-2003, is an important safeguard against future outbreaks. Two cysteine proteases that reside within the polyprotein, the PLpro and a 3C-like protease, catalyze their own release and that of other nonstructural proteins from the polyprotein, thereby initiating virus-mediated RNA replication. The numerous functions and requisite roles of PLpro in viral replication and pathogenesis suggest that it may serve as an attractive target for antiviral drugs. A limited screen of 50,080 compounds for inhibitors of PLpro was employed using a fluorescent substrate based on the C terminal-pentapeptide of ubiquitin, Z-RLRGG-AMC. Of the original 17 hits, 9 compounds were found to interfere with the fluorescence of the AMC reporter group (4). The high background signal associated with the conjugated fluorophores and interference with library compounds reinforces the need for additional assays. The DUB-Glo™ Protease Assay provides a more sensitive, bioluminescent alternative to fluorescent assays for measuring the deubiquitinating enzyme activity of the SARS-CoV PLpro (Figure 3). The bioluminescent assay displays a stable and broader linear range with low background signal, where light intensity is clearly proportional to deubiquitinating activity (Figures 3 and 4). The assay produces robust signal even with low levels of SARS CoV PLpro in a 384-well format (Figure 4).

The DUB-Glo™ Protease Assay was tested with a well-characterized inhibitor of SARS CoV PLpro. The results demonstrated dose-dependent inhibition of the PLpro enzymatic activity with an IC_{50} that corresponded to a previously determined K_i for this inhibitor; Figure 5; A. Mesecar, unpublished results). The sensitivity of the bioluminescent assay enabled using the Z-RLRGG-aminoluciferin substrate at a concentration well-below the K_m; under these conditions, the IC_{50} for a competitive inhibitor is the

same as the K_i[17] . The previous fluorescent screen for inhibitors of SARS-CoV PLpro used the Z-RLRGG-AMC substrate at a 50-fold higher concentration (50µM)[15] .

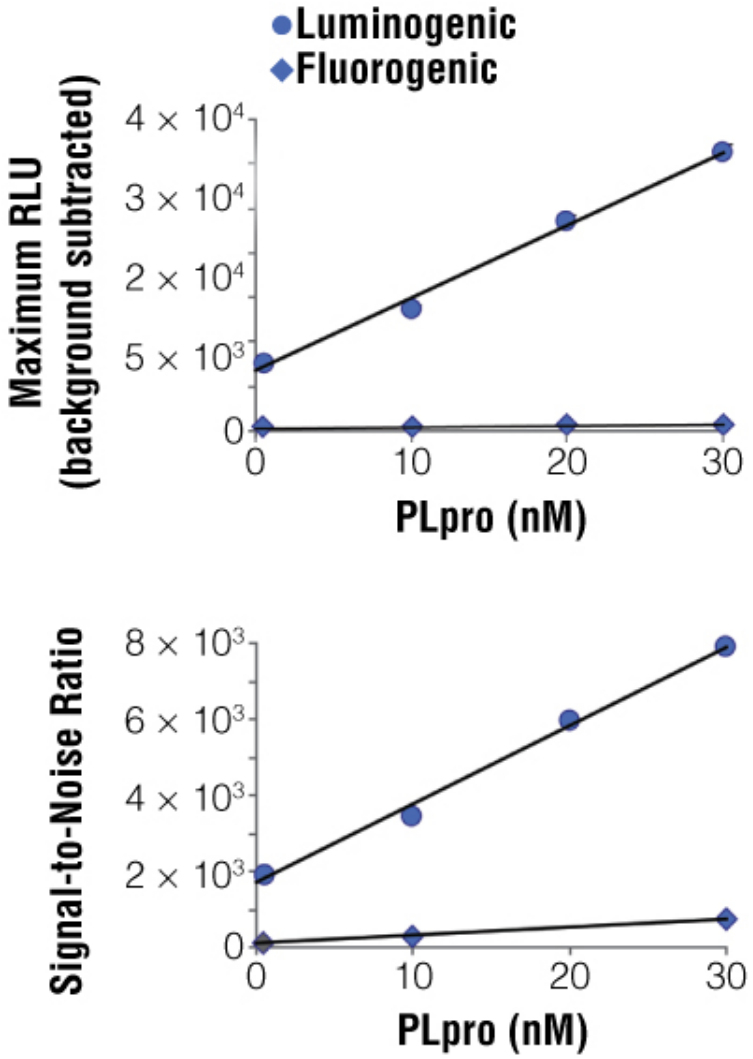


Figure 3. A Comparison of the DUB-Glo™ Protease Assay to the AMC-based Fluorescent Assay. The SARS-CoV PLpro was diluted in 50mM HEPES (pH 7.5), 5mM DTT, and 0.1mg/ml BSA and combined with the DUB-Glo™ Reagent or the Z-RLRGG-AMC substrate in 96-well plates (100µl total volume). The bioluminescent assay gives a broader dynamic range and the signal-to-noise ratios showed an increase in sensitivity of approximately 1O-fold for the DUB-Glo™ Protease Assay.

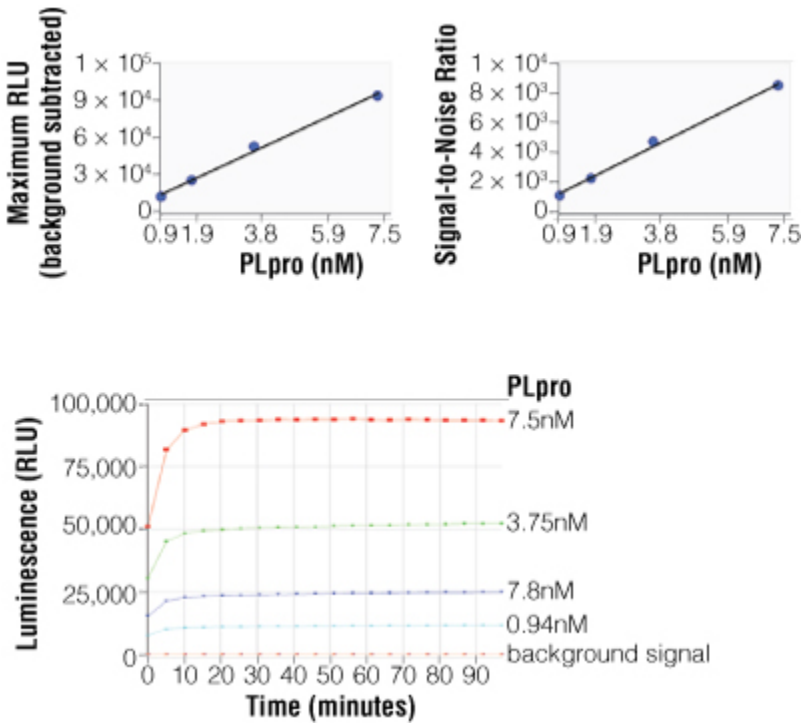
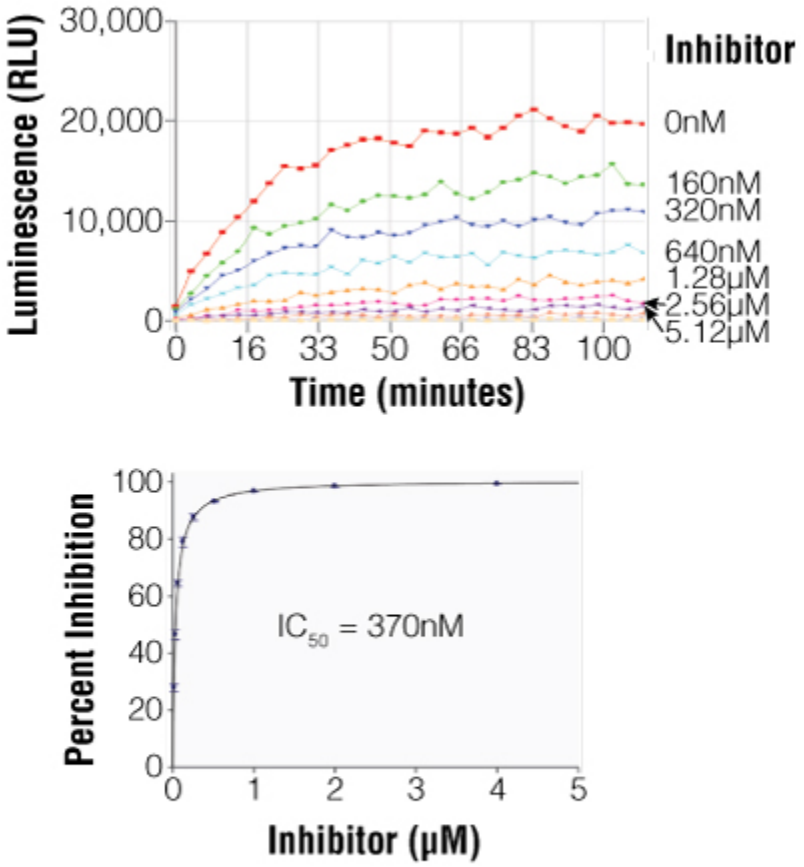


Figure 4. DUB-Glo™ Protease Assay Time Course with Varying Concentrations of SARS-CoV PLpro. The PLpro was diluted in 50mM HEPES (pH 7.5), 5mM DTT, and 0.5mM EDTA and combined with the DUB-Glo™ Reagent in 384-well plates (50µl total volume). The final concentration for the Z-RLRGG-aminoluciferin substrate was 1µM. The assay gives stable signal, low background in a miniaturized format.



Maximum Percent Inhibition	IC_{50}	Z Factor
100±0.6	0.4±0.01	0.71

Figure 5. Dose-dependent inhibition of SARS-CoV PLpro activity using the DUB-Glo™ Protease Assay. The SARS-CoV PLpro was added to a titration of a competitive inhibitor of SARS-CoV in 50mM HEPES (pH 7.5), 5mM DTT, and 0.1mg/ml BSA and combined with the DUB-Glo™ Reagent in 96-well plates (100µl total volume). The final concentration for the Z-RLRGG-aminoluciferin substrate was 1µM and the SARS-CoV PLpro was 30nM.

Summary

Typical fluorogenic substrates for deconjugating proteases based on the full-length sequence of the modifier (e.g. Ubiquitin-AMC, Sumo-AMC, and NEDD8-AMC) are not without limitations. Substrate depletion, substrate inhibition, and general issues of fluorescence background and interference are particularly problematic for high throughput screening. The homogeneous, bioluminescent DUB-Glo™ Protease Assay is the first peptide-based assay that has the sensitivity necessary for monitoring the activity of several deubiquitinating, desumoylating, and deneddylating proteases. The assay is designed as a coupled-enzyme format that has very low background with high signal-to-background ratios. It gives very stable light output with maximum sensitivity reached quickly, and is very flexible, making it ideal for high-throughput screening applications. The assay was tested with the SARS-CoV PLpro and shown to be significantly more sensitive than a comparable fluorescent assay. This enables using the substrate and protease at lower concentrations while still retaining a broad linear dynamic range. A test inhibitor gave the predicted results, demonstrating the usefulness of the DUB-Glo™ Protease Assay for inhibitor screening.

The luminogenic peptide-based substrate avoids inherent issues with full-length substrates and provides a novel alternative to standard AMC-conjugated substrates.

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HOW TO CITE THIS ARTICLE

Scientific Style and Format, 7th edition, 2006

Baez-Santos, Y. M., Mesecar, A. D. and O'Bien, M. A. Detecting the Deubiquitin Activity of SARS-CoV PLpro: Turning on the Light with the DUB-Glo™ Protease Assay . [Internet] 2009. [cited: year, month, date]. Available from: http://nld.promega.com/resources/pubhub/detecting-the-deubiquitin-activity-of-sars-cov-plpro-turning-on-the-light-with-the-dub-glo/

American Medical Association, Manual of Style, 10th edition, 2007

Baez-Santos, Y. M., Mesecar, A. D. and O'Bien, M. A. Detecting the Deubiquitin Activity of SARS-CoV PLpro: Turning on the Light with the DUB-Glo™ Protease Assay . Promega Corporation Web site. http://nld.promega.com/resources/pubhub/detecting-the-deubiquitin-activity-of-sars-cov-plpro-turning-on-the-light-with-the-dub-glo/ Updated 2009. Accessed Month Day, Year.

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