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Technical Bulletin

Calpain-Glo™ Protease Assay

INSTRUCTIONS FOR USE OF PRODUCTS G8501 AND G8502.

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Calpain-Glo™ Protease Assay

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1. Description

The Calpain-Glo™ Protease Assay^(a,b) is a homogeneous, luminescent assay that measures calpain I (µ) and II (m) activities. Calpains are a family of calcium-activated cysteine proteases involved in cleaving a wide variety of proteins. They function in Ca²⁺ signaling by modulating biological activities of their substrates through limited proteolysis (1). Calpains have been implicated in numerous calcium-regulated cellular processes including: cell motility, cell cycle progression, cell proliferation, apoptosis, necrosis, differentiation, membrane fusion and platelet activation (1-4). Calpains are also thought to be involved in numerous pathological conditions, making them important therapeutic targets (5,6). Deregulated calpain activity following loss of calcium homeostasis results in tissue damage in response to events such as myocardial infarctions, stroke and brain trauma (2). Defining physiological substrates for calpains has been elusive. The rapid inactivation of calpains upon calcium activation makes them difficult to work with and limits the sensitivity and usefulness of traditional fluorometric and colorimetric assays.

The Calpain-Glo™ Protease Assay provides a succinyl proluminescent calpain substrate, Suc-LLVY-aminoluciferin, in a buffer system optimized for calpain activity and luciferase activity. Adding the single Calpain-Glo™ Reagent to test samples in an “add-mix-measure” format results in calpain cleavage of the substrate and generation of 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 calpain activity present (Figure 2).

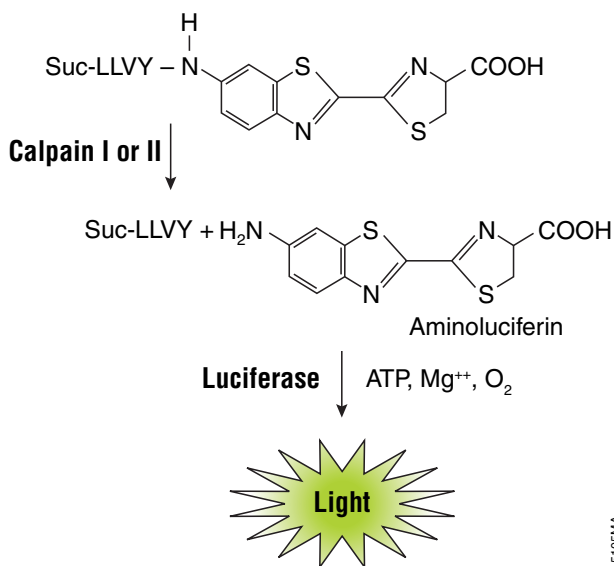


Figure 1. The proluminescent substrate containing the Suc-LLVY sequence recognized by calpain. Following calpain cleavage, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to occur and producing light.

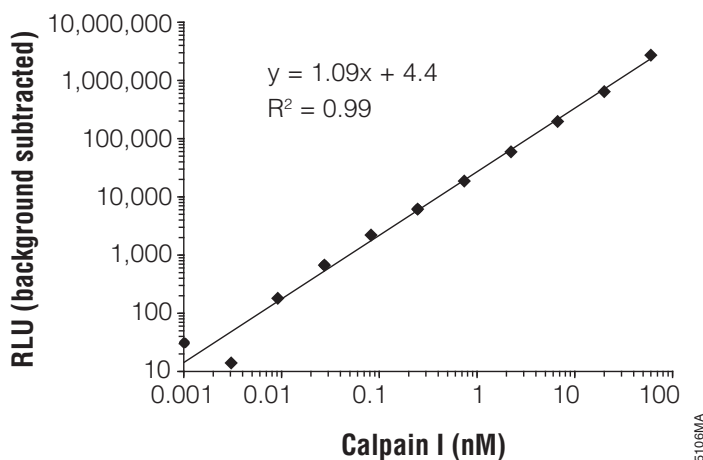


Figure 2. Luminescence is proportional to calpain activity. A titration assay of calpain was performed in 96-well plates using the Calpain-Glo™ Protease Assay. Human Calpain I was serially diluted in 10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA + 0.1% Prionex® as a carrier. Twelve minutes after addition of the Calpain-Glo™ Reagent, luminescence was recorded as relative light units (RLU) on a GloMax™ 96 Microplate Luminometer. The results were linear over 4 logs of calpain concentration ($R^2 = 0.99$, slope = 1.09). Each point represents the average of 4 wells. The background (blank without calpain) was subtracted from each (average blank RLU = 885). R^2 and slope were calculated after transforming the data to a \log_{10} - \log_{10} plot.

The Calpain-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (UltraGlo™ recombinant luciferase) that is formulated to generate a “glow-type” luminescent signal and improve performance across a wide range of assay conditions. The calpain and luciferase enzyme activities reach a steady-state so that the luminescent signal peaks rapidly and achieves maximum sensitivity in 5–15 minutes. The signal then decays as the calpain enzyme becomes inactive (Figure 3). The Calpain-Glo™ Protease Assay provides a rapid, sensitive, and accurate calpain activity assay (Figures 4 and 5). The homogeneous Calpain-Glo™ Assay is designed for use with multiwell plate formats making it ideal for automated high-throughput screening (HTS) of calpain activity and inhibition (Figure 6).

Assay Advantages

Broad Dynamic Range: The assay is linear over 4 logs of calpain I concentrations and can detect calpain I at concentrations as low as 5pM (Figures 2 and 4).

Fast: Maximum sensitivity is reached in 5–15 minutes after reagent addition (Figure 3), since the assay is not dependent on accumulation of cleaved product for sensitivity.

Greater Sensitivity: The coupled-enzyme format and the speed of the Calpain-Glo™ Assay results in very low background and excellent signal-to-noise ratios. The assay is significantly more sensitive than fluorescent-based calpain assays (Figure 4).

Simplified Method: The homogeneous “add-mix-read” protocol makes the assay amenable to automation (Figure 6).

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

Amenable to Batch Processing: The coupled-enzyme, homogeneous format results in a glow-type signal, allowing flexibility in read time once the reagent is added (Figure 3A and 4).

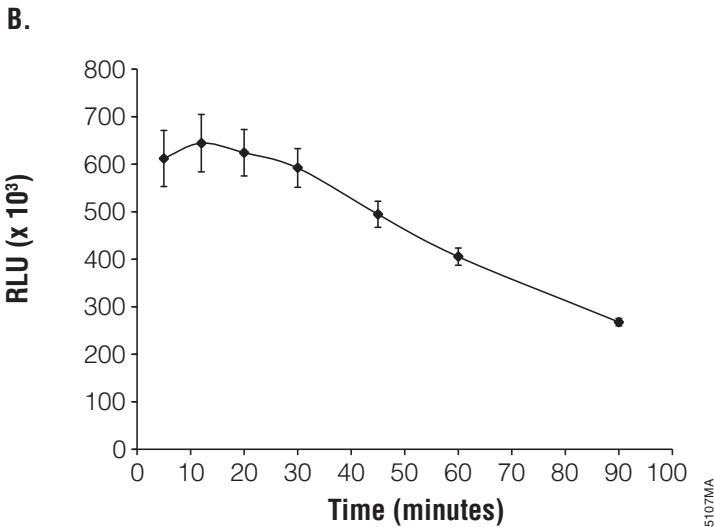
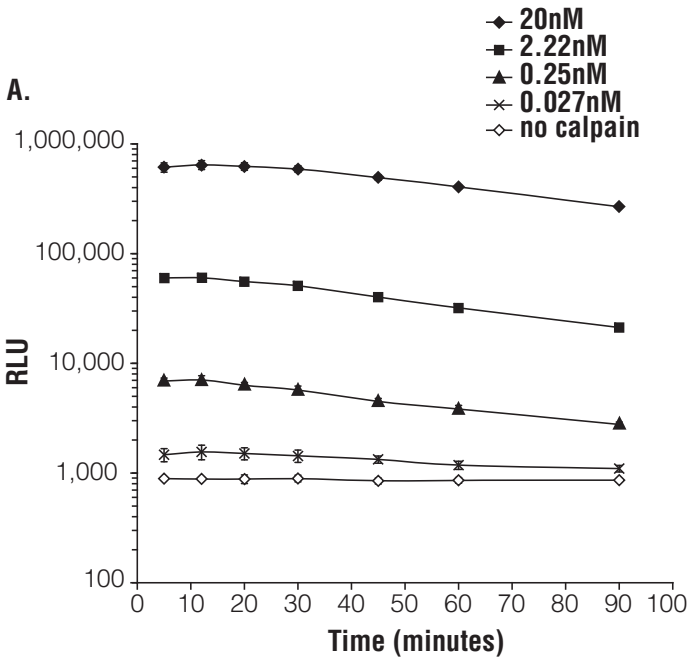


Figure 3. Signal half-life of the Calpain-Glo™ Protease Assay. Human calpain I from plasma was titrated and assayed in 96-well plates using the Calpain-Glo™ Assay. Luminescence was monitored at various times for 1.5 hours on a GloMax™ 96 Microplate Luminometer. **Panel A.** The signal peaks rapidly and then decreases uniformly over a broad calpain concentration. The signal decrease is primarily a result of the inactivation of calpain upon calcium activation. **Panel B.** The luminescent signal has a half-life of >60 minutes as shown for the 20nM calpain concentration.

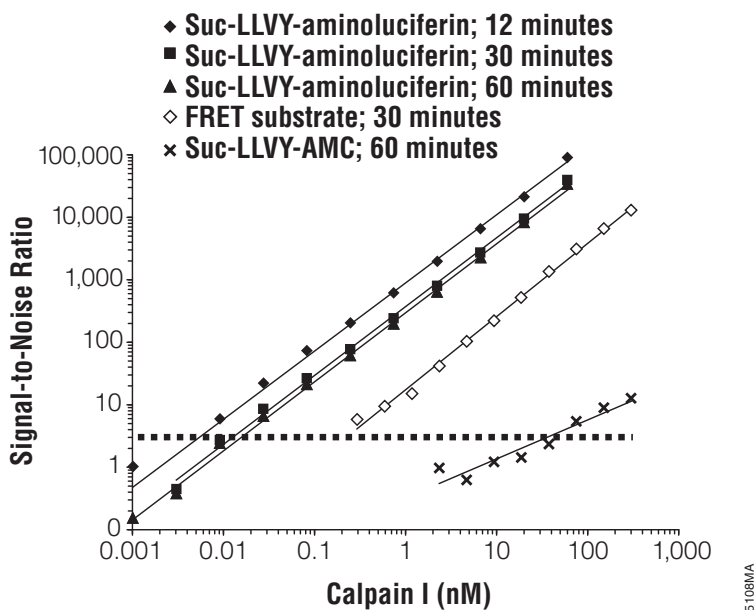


Figure 4. Sensitivity of the Calpain-Glo™ Protease Assay compared to fluorescent calpain assays. Human calpain I was titrated and assayed in 96-well plates using the Calpain-Glo™ Assay, a Suc-LLVY-AMC fluorescent substrate, or the FRET-based substrate, [H-Lys (FAM)-EVYGMK(Dabcyl)-OH]. Luminescence was recorded at various times after reagent addition on a GloMax™ 96 Microplate Luminometer. Fluorescence was monitored on a LabSystems Ascent fluorometer. The results are plotted as signal-to-noise ratios (7). The limit of detection is defined as the amount of calpain giving a signal-to-noise ratio=3 (dashed line). The bioluminescent assay demonstrated a limit of detection of 5pM in 12 minutes whereas the FRET-based fluorescent assay demonstrated a limit of detection of only 200pM after 30 minutes and the Suc-LLVY-AMC substrate gave a limit of detection of 30nM after 60 minutes (earlier time points did not result in fluorescence above background).

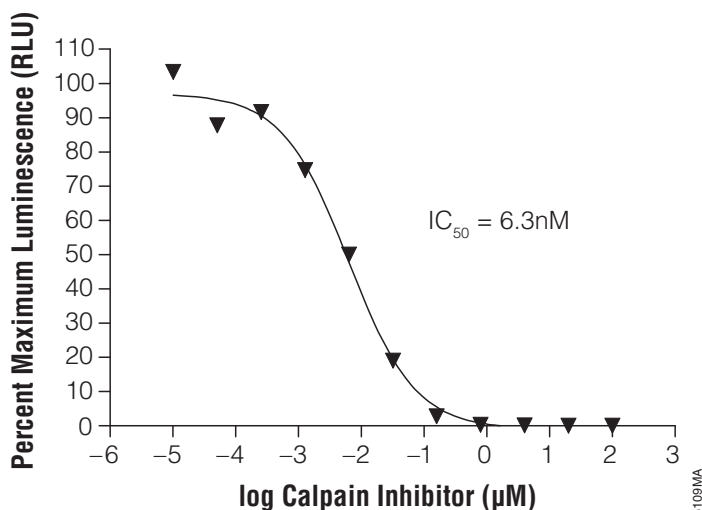


Figure 5. Determination of IC₅₀ values. The concentration of inhibitor that results in 50% inhibition (IC₅₀) was determined for the calpain competitive inhibitor, Z-Val-Phe-CHO (inhibitor III), using the Calpain-Glo™ Protease Assay. The inhibitor was resuspended in DMSO, serially diluted and combined with human calpain I (20nM) in 10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA, 1mM EGTA + 0.1% Prionex® in 96-well plates. The maximum DMSO concentration is 0.1%. The Suc-LLVY-Glo™ substrate was used at the apparent K_m. Luminescence was recorded 10 minutes after reagent addition and GraphPad Prism® software was used to calculate the IC₅₀ of 6.3nM. This corresponds closely to the K_i provided by Calbiochem (8nM; Cat.# 208722).

2. Product Components

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml	G8501
	50ml	G8502

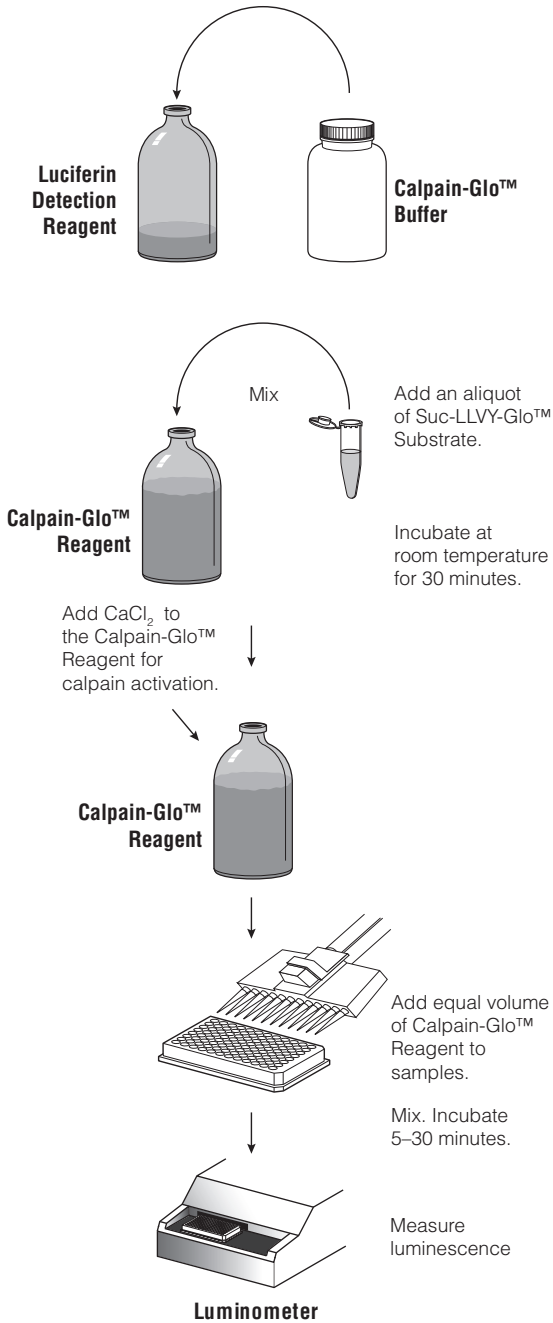
Cat.# G8501 is sufficient for 100 assays at 100µl/assay or 200 assays at 50 µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 10ml Calpain-Glo™ Buffer
- 100µl Suc-LLVY-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Cat.# G8502 is sufficient 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 Calpain-Glo™ Buffer
- 500µl Suc-LLVY-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Storage Conditions: Store the Calpain-Glo™ Assay components at -20°C protected from light. The Calpain-Glo™ Buffer may be thawed and stored at 4°C for 2 months with no loss in signal. The Suc-LLVY-Glo™ Substrate may be refrozen and stored at -20°C with minimal loss of signal. Calpain-Glo™ Reagent (combined Suc-LLVY-Glo™ Substrate, Calpain-Glo™ Buffer, Luciferin Detection Reagent and CaCl₂) can be stored at 4°C or -20°C for 1 month with minimal loss of activity.



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

3. Reagent Preparation

Directions are given for performing the Calpain-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 providing the 1:1 ratio of Calpain-Glo™ Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl Calpain-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's will be reduced)
- multichannel pipette or automated pipetting station for delivery of Calpain-Glo™ Reagent
- plate shaker for mixing multiwell plates
- luminometer capable of reading multiwell plates
- calpain enzyme (e.g., Biovision Cat.# 1134-100 or Calbiochem Cat.# 208713)
- calcium chloride (CaCl₂; 1M stock recommended)

Calpain-Glo™ Reagent Preparation

1. Thaw the Calpain-Glo™ Buffer and equilibrate both Buffer and the lyophilized Luciferin Detection Reagent to room temperature prior to use.
2. Reconstitute the Luciferin Detection Reagent in the amber bottle by adding the appropriate volume of Calpain-Glo™ Buffer (10ml for Cat.# G8501, 50ml for Cat.# G8502). The Luciferin Detection Reagent should go into solution easily in less than one minute.
3. Thaw the Suc-LLVY-Glo™ Substrate and equilibrate to room temperature prior to use. A slight precipitate may be observed. **Mix well by vortexing briefly.**
4. Prepare the Calpain-Glo™ Reagent by adding the Suc-LLVY-Glo™ Substrate to the resuspended Luciferin Detection Reagent. For Cat.# G8501, add 100µl of Suc-LLVY-Glo™ Substrate to the 10ml of Luciferin Detection Reagent. For Cat.# G8502, add 500µl of Suc-LLVY-Glo™ Substrate to the 50ml of Luciferin Detection Reagent. Mix to homogeneity by swirling or inverting the contents. The Suc-LLVY-Glo™ Substrate will be at a concentration of 80µM in the Calpain-Glo™ Reagent and 40µM in the final assay. The apparent K_m for the substrate is 40µM.
5. **Allow the Calpain-Glo™ Reagent to sit at room temperature for 30 minutes prior to use.** This allows for the removal of any contaminating free aminoluciferin. Although free aminoluciferin is not detected by HPLC, it is present in trace amounts (Figure 7).

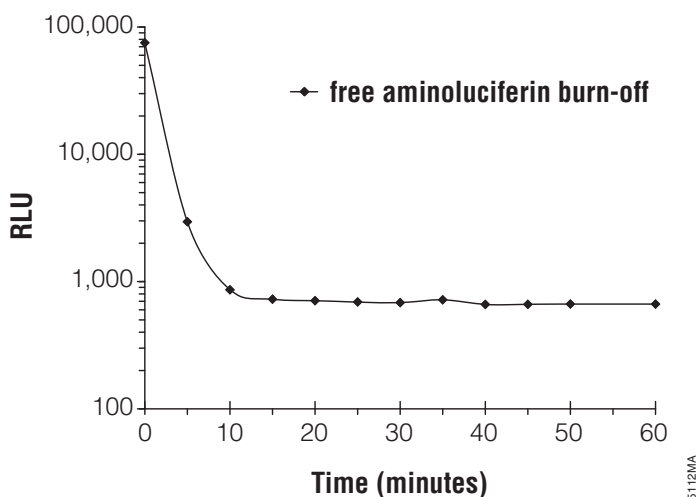


Figure 7. Time course of free aminoluciferin removal from the Calpain-Glo™ Reagent. The calpain substrate (Suc-LLVY-aminoluciferin) was added to the reconstituted Luciferin Detection Reagent, and a time course of luminescence was recorded. Trace amounts of free aminoluciferin are present in the substrate and can be “burned-off” by incubation with the reconstituted Luciferin Detection Reagent. To achieve maximal assay sensitivity with minimal background luminescence, the prepared Calpain-Glo™ Reagent should be incubated for 30 minutes before use.

6. Add CaCl₂ to the Calpain-Glo™ Reagent prior to adding to calpain test wells if calpain activation is required. The final recommended concentration in the reagent should be 2mM CaCl₂ (1mM in final assay volume). For Cat.# G8501, add 20µl of 1M CaCl₂ to the 10ml of Calpain-Glo™ Reagent. For Cat.# G8502, add 100µl of 1M CaCl₂ to the 50ml of Calpain-Glo™ Reagent.

4. Assay for Detection of Calpain Activity

4.A. Assay Conditions

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

- **Blank:** Calpain-Glo™ Reagent + CaCl₂ (2mM) + vehicle control for enzyme test sample or inhibitor, if used.
- **Positive Control:** Calpain-Glo™ Reagent + CaCl₂ (2mM) + vehicle control + purified calpain enzyme.
- **Assay:** Calpain-Glo™ Reagent + CaCl₂ (2mM)+ test sample + purified calpain enzyme.

The blank is used as a measure of any background luminescence associated with the test sample vehicle and Calpain-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 sample used in the study. Calpain-Glo™ Reagent without CaCl₂ can be used as an additional negative control with calpain enzyme samples.

Notes:

1. Prepare the Calpain-Glo™ Reagent as described in Section 3 and mix thoroughly prior to starting the assay.
2. The final concentration of calpain enzyme should be 50nM or less.
3. The recommended calpain enzyme dilution buffer is 10mM HEPES (pH 7.2), 10mM DTT and 1mM EDTA. Use a carrier such as 0.1% Prionex® or BSA if low enzyme quantities are being used.
4. Use identical enzyme concentrations for the assay and positive control reactions.
5. Gentle mixing may be done using a plate shaker.
6. The maximal luminescent signal will be reached in 5–15 minutes and will have a half-life of approximately 30–70 minutes (Figure 3).

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

1. Add 50µl of Calpain-Glo™ Reagent (with 2mM CaCl₂ added for calpain activation) 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 5–30 minutes depending upon convenience of reading time (Figure 3, Panel A). Maximal signal is typically reached within 5–15 minutes using calpain I enzyme (Figure 3, Panel B). At this time, sensitivity is optimal. Temperature fluctuations will impact 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 Calpain-Glo™ Protease Assay is more sensitive than comparable fluorescent assays for several reasons. The luminescent substrate (Suc-LLVY-aminoluciferin) is not a substrate for luciferase until it is 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 calpain substrate 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 allows for a very broad linear range for the assay (4 logs of calpain concentration; Figures 2 and 4). The assay sensitivity allows the researcher to use less enzyme if screening for calpain inhibitors. We recommend using no more than 50nM calpain; at higher concentrations, the kinetics of the assay are compromised due to product inhibition of the luciferase reaction.

This coupled-enzyme assay is not dependent on accumulation of cleaved product because the light output is a result of luciferase consuming the aminoluciferin substrate as soon as it is produced by the protease. Maximum sensitivity is achieved as soon as the calpain and luciferase activities reach a steady-state. Typically this occurs in 5–15 minutes; therefore, the assay is extremely sensitive in a short time frame.

Signal Half-Life

The Calpain-Glo™ Assay signal is directly proportional to the calpain activity; therefore, as the calpain enzyme becomes inactivated after calcium activation, the luminescent signal decreases. Figure 3 shows a typical time course for the signal decay over a broad concentration of human calpain I. The half-life for the signal is typically 30–70 minutes, but there are many factors that will affect the inactivation rate of calpain including temperature, ionic strength, pH, calcium concentration and the source of the enzyme preparation. The reagent has been optimized to minimize these variables, but the signal half-life may vary depending on the calpain preparation used and the temperature of the laboratory .

Chemicals

The chemical environment of the calpain and luciferase reaction will affect the enzymatic rate and luminescence intensity. It is possible that solvents used for various chemical compounds may interfere with the calpain and luciferase reactions and 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 10% in the assay (Figure 8). Concentrations up to 2.5% DMSO had minimal effects on the assay. The signal was decreased by 26% with 5% DMSO, and 10% DMSO increased the time to peak signal to 40 minutes and decreased the signal by 54%. Inhibitors detected in a screen using the Calpain-Glo™ Assay can be ruled out as luciferase inhibitors by testing with luciferin and the Luciferin Detection Reagent.

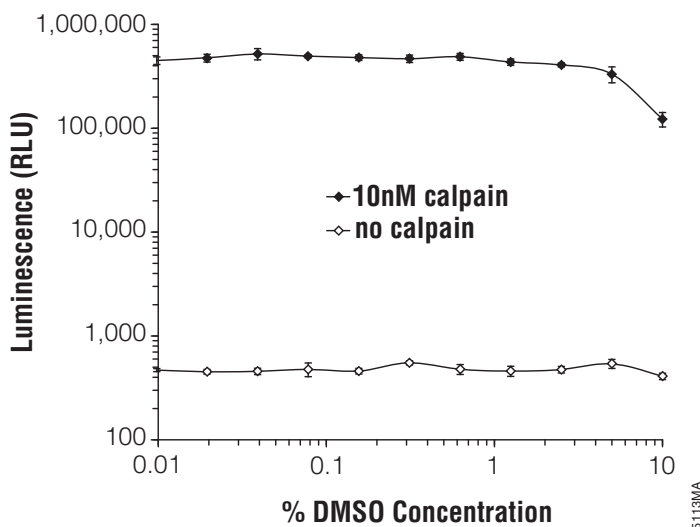


Figure 8. DMSO effects on Calpain-Glo™ Assay. DMSO was titrated and combined with human calpain I (10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA, 1mM EGTA + 0.1% Prionex®) in 96-well plates (50µl/well). The Calpain-Glo™ Reagent + 2mM CaCl₂ was added and luminescence was recorded after 10 minutes on a GloMax™ 96 Microplate Luminometer.

Mixing

Mixing is not absolutely required after adding the Calpain-Glo™ Reagent, although it may aid in reproducibility among wells.

6. References

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5. Chen, M. and Fernandez, H.L. (2005) mu-Calpain is functionally required for alpha-processing of Alzheimer's beta-amyloid precursor protein. *Biochem. Biophys. Res. Commun.* **330**, 714-21.
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7. Related Products

Product	Size	Cat.#
DPPIV-Glo™ Protease Assay	10ml	G8350
	50ml	G8351
Caspase-Glo® 3/7 Assay	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Caspase-Glo® 8 Assay	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay	2.5ml	G8210
	10ml	G8211
	100ml	G8212
Apo-ONE® Homogeneous Caspase-3/7 Assay (fluorescent)	1ml	G7792
	10ml	G7790
	100ml	G7791
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573

7. Related Products (continued)

Product	Size	Cat.#
CellTiter-Blue® Cell Viability Assay	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
	1,000–4,000 assays	G7891
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774
P450-Glo™ CYP1A1 Assay	10ml	V8751
	50ml	V8752
P450-Glo™ CYP1B1 Assay	10ml	V8761
	50ml	V8762
P450-Glo™ CYP1A2 Assay	10ml	V8771
	50ml	V8772
P450-Glo™ CYP2C8 Assay	10ml	V8781
	50ml	V8782
P450-Glo™ CYP2C9 Assay	10ml	V8791
	50ml	V8792
P450-Glo™ CYP3A4 Assay	10ml	V8801
	50ml	V8802
P450-Glo™ CYP3A7 Assay	10ml	V8811
	50ml	V8812
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
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-Glycoprotein	10ml	V3601
GloMax™ 96 Microplate Luminometer		E6501

^(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. Nos. 6,602,677 and 7,241,584, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

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