Technical Bulletin

Caspase-Glo® 6 Assay

INSTRUCTIONS FOR USE OF PRODUCTS G0970 AND G0971.

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Caspase-Glo® 6 Assay

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1. Description

The Caspase-Glo® 6 Assay[a,b,c] is a homogeneous, luminescent assay that measures caspase-6 activity. Caspase-6 is a member of the cysteine aspartic-acid-specific protease (caspase) family and has a key effector role in the cleavage of specific target proteins during apoptosis (1–3). Caspases involved in apoptosis are divided into initiator and effector caspases. Initiator caspases function upstream within apoptotic signaling pathways, and effector caspases are downstream executioners of the apoptotic program, cleaving several substrates to cause controlled breakdown of the cell. Along with caspases-3 and -7, caspase-6 is one of the effector caspases of apoptosis, functioning downstream of the initiator caspases and cytochrome c release from the mitochondria (4). Changes in caspase-6 activity have been associated with neurogenerative diseases such as Huntington disease and Alzheimer disease (3,5–8).

The Caspase-Glo® 6 Assay provides a luminogenic caspase-6 substrate, Z-VEID-aminoluciferin, in a buffer system optimized for caspase-6 and luciferase activities. The addition of a single Caspase-Glo® 6 Reagent in an "add-mix-measure" format results in caspase-6 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 luminescent signal is proportional to the amount of caspase-6 activity present (Figure 2). The Caspase-Glo® 6 Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a stable glow-type luminescent signal across a wide range of assay conditions.

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The homogeneous Caspase-Glo® 6 Assay is designed for use with multiwell plate formats, making it ideal for automated high-throughput screening for caspase-6 activity and inhibitors of caspase-6 activity. The caspase-6 and luciferase enzyme activities reach a steady state so that the luminescent signal peaks rapidly and is maintained for several hours with minimal loss of signal (Figure 3). This provides a rapid, sensitive and flexible caspase-6 activity assay. This luminescent format significantly improves the sensitivity over comparable fluorescent assays (Figure 5). The Caspase-Glo® 6 Buffer contains a lysis reagent that allows measurement of caspase activities in cultured cells, but cross-reactivity of the Z-VEID-aminoluciferin substrate with other caspases must be considered and may complicate data interpretation in cell-based assays (Figure 7). Thus, the Caspase-Glo® 6 Assay has been developed for use with purified enzyme preparations or for inhibitor screens (Figures 2 and 6).

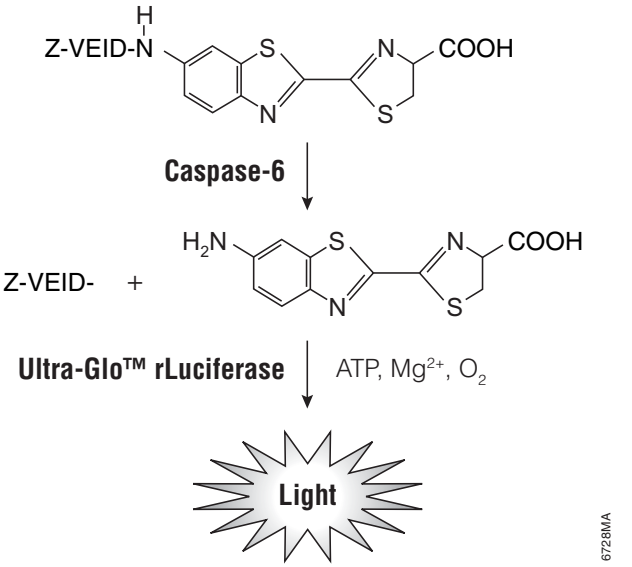

Figure 1. Caspase-6 cleavage of the luminogenic substrate containing the VEID consensus sequence. Following caspase-6 cleavage, the substrate for luciferase, aminoluciferin, is released, resulting in the production of light from the luciferase reaction.

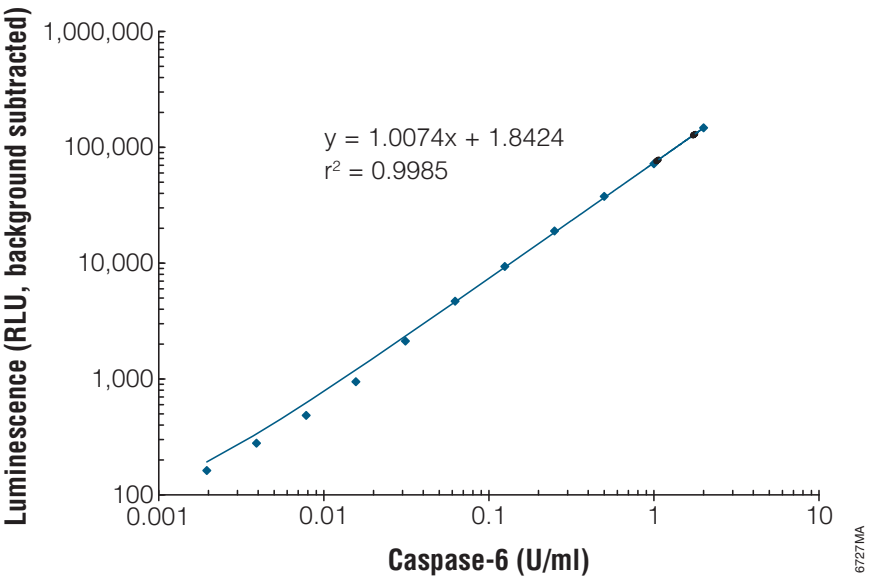

Figure 2. Titration of caspase-6 enzyme assayed in 96-well plates using the Caspase-Glo® 6 Assay. Recombinant human caspase-6 (BIOMOL International) was serially diluted in HEPES buffer [10mM HEPES (pH 7.2), 2mM DTT, 0.1% Prionex® carrier]. Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer (Cat.# E6501) 30 minutes after adding the Caspase-Glo® 6 Reagent. The assay is linear over 3 logs of caspase-6 concentration (r^2 = 0.9985, slope = 1.0074). Each point represents the average of 4 wells. The no-caspase-6 control value was subtracted from each average. Values for r^2 and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot. One unit of caspase-6 was defined as the amount of enzyme required to cleave 1pmol of substrate/minute at 30°C using 200µM of Ac-VEID-pNA as substrate.

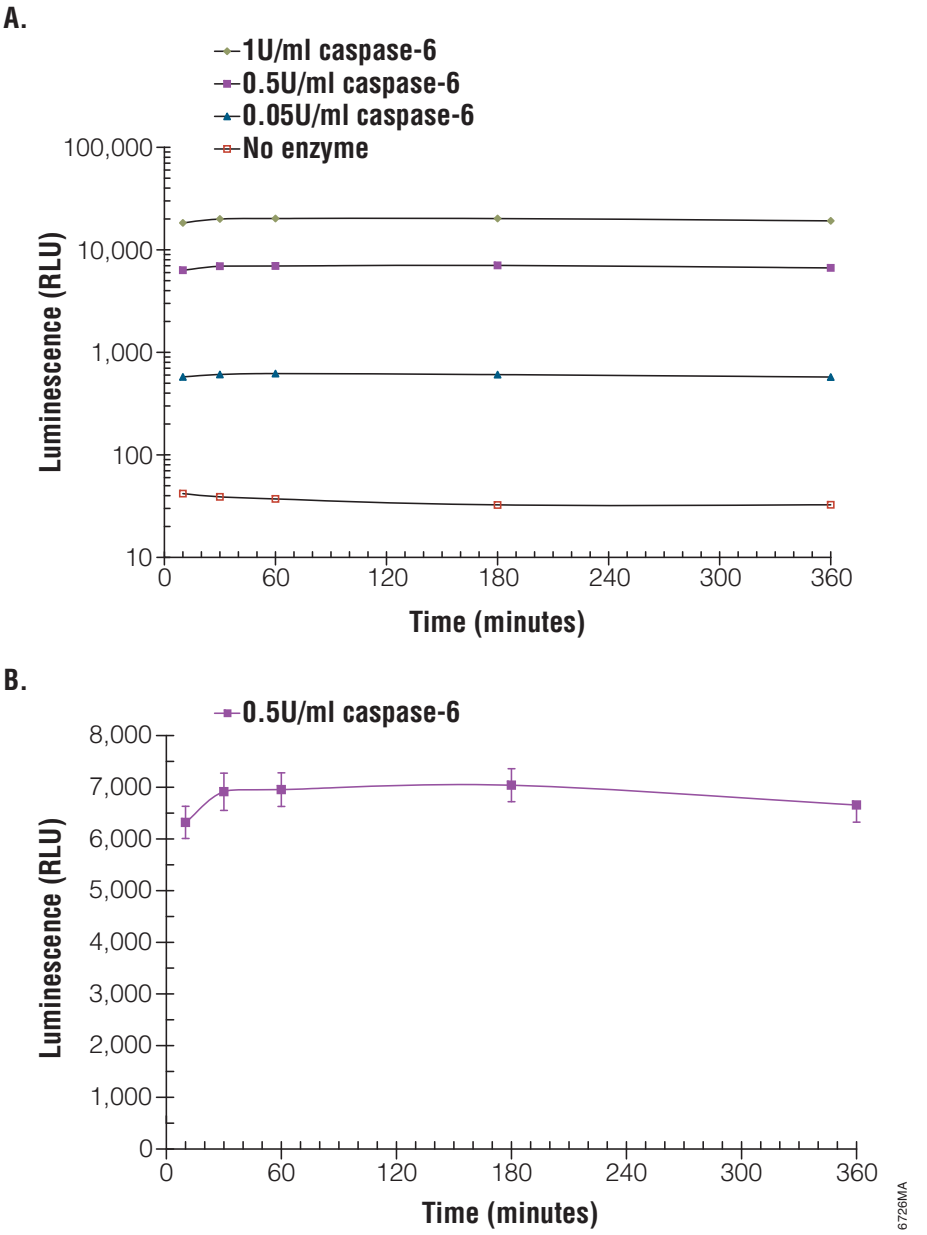

Figure 3. Signal stability of the Caspase-Glo® 6 Assay. Recombinant human caspase-6 enzyme (BIOMOL International) was titrated and assayed in a 384-well plate using the Caspase-Glo® 6 Assay. Luminescence was monitored for 6 hours on a BMG FLUOstar multimode reader. **Panel A.** The assay gives a stable signal for several hours over a broad range of caspase-6 concentration (shown on a log scale). **Panel B.** The luminescent signal has a half-life greater than 6 hours, shown here for the 0.5U/ml caspase-6 concentration (linear scale).

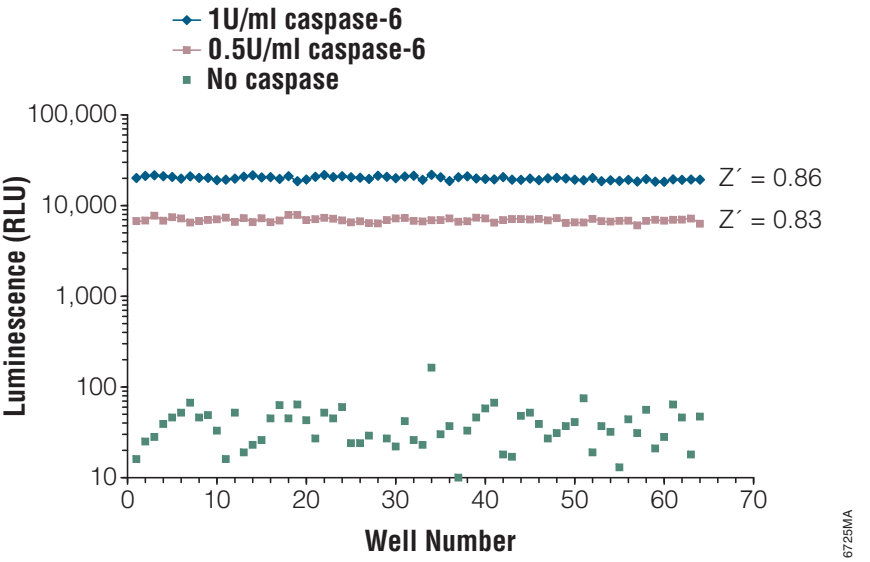

Figure 4. Z´-factor value analysis of the Caspase-Glo® 6 Assay. Z´-factor values were calculated using 1U/ml and 0.5U/ml recombinant caspase-6 (BIOMOL International) and a no-caspase blank. Assays were performed in a 384-well plate using the Caspase-Glo® 6 Assay. Luminescence was recorded as relative light units (RLU) on a BMG FLUOstar multimode reader 30 minutes after adding the Caspase-Glo® 6 Reagent.

Advantages of the Caspase-Glo® 6 Assay

Simplified Method: The homogeneous "add-mix-measure" protocol makes the assay highly amenable to automation (Figure 9).

Greater Sensitivity: The assay is more sensitive than fluorescence-based caspase-6 assays. In contrast to fluorescent assays, the luminescent assay avoids inherent fluorescent background signals, providing excellent signal-to-noise ratios. The assay is linear for 3 logs of caspase-6 concentration and can detect 0.002U/ml (Figures 2 and 5).

Faster Results: The maximum signal (and maximum sensitivity) of the assay is reached in as little as 30 minutes after reagent addition (Figure 3, Panel B), and unlike fluorescent assays, signal is not dependent on accumulation of cleaved product.

Increased Accuracy: The assay provides accurate results for kinetic studies of inhibitors (Figure 6). Because of the sensitivity of the Caspase-Glo® 6 Assay, inhibitor studies can be done using the luminescent substrate at concentrations significantly below the K_m. In the case of competitive inhibitors, if the substrate is used at concentrations significantly below the K_m, the $IC_{50} = K_i$ (9).

Assay Advantages (continued)

High-Quality Assay: The assay demonstrates an excellent Z´-factor value, a statistical value that compares the dynamic range of an assay to data variation. Z´-factor values >0.5 indicate excellent assay quality (10). The Caspase-Glo® 6 Assay gave a Z´-factor value of 0.86 and 0.83 when using 1U/ml and 0.5U/mL of caspase-6, respectively, for assays in 384-well plates (Figure 4).

Batch-Processing Capability: The coupled-enzyme, homogeneous format results in a continuous signal, providing excellent stability and allowing plates to be read over an extended period of time (Figure 3, Panel A).

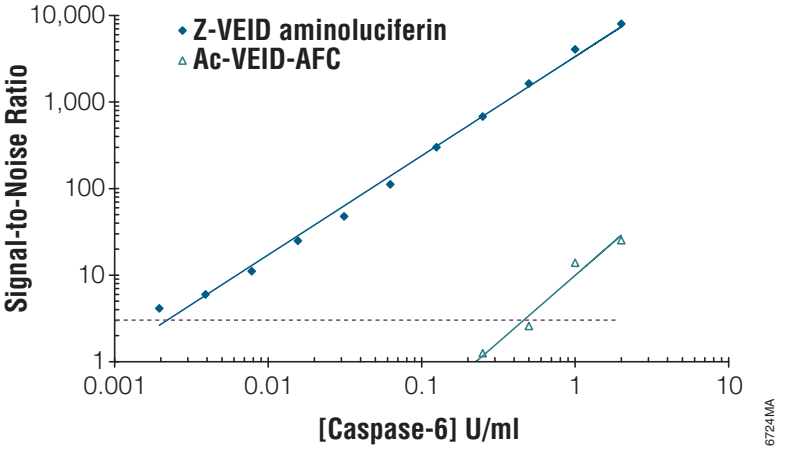

Figure 5. Sensitivity of the Caspase-Glo® 6 Assay compared to a fluorescent caspase-6 assay. Recombinant caspase-6 enzyme was titrated and assayed in 96-well plates using the Caspase-Glo® 6 assay or an Ac-VEID-AFC fluorescent substrate. Luminescence and fluorescence were monitored after 60 minutes on a BMG FLUOstar multimode reader. The results are plotted as signal-to-noise ratios. The limit of detection is defined as the amount of caspase-6 giving a signal-to-noise ratio >3 (dashed line). The bioluminescent assay demonstrated a limit of detection of ~0.002U/ml, whereas the fluorescent assay demonstrated a limit of detection of ~0.5U/ml after 60 minutes.

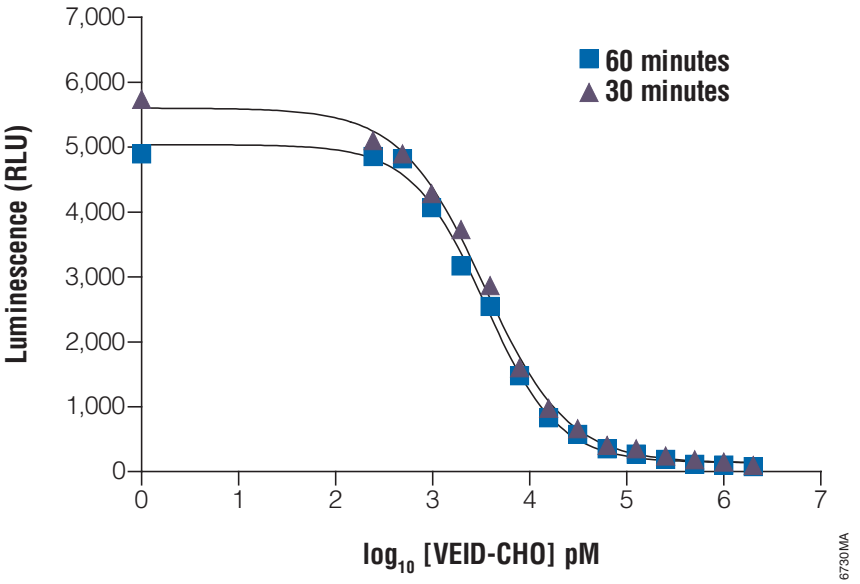

Figure 6. Determination of IC_{50} values. The inhibitor concentration that results in 50% inhibition (IC_{50}) was determined for the caspase-6 competitive inhibitor, Ac-VEID-CHO, using the Caspase-Glo® 6 Assay. The inhibitor was resuspended in DMSO and serially diluted in HEPES buffer (10mM HEPES [pH 7.2], 2mM DTT and 0.1% Prionex® as a carrier) and combined with 0.25U/ml of caspase-6 in 96-well plates. The maximum DMSO concentration was 0.01%. The caspase-6 substrate was used at 0.5µM, a concentration well below the apparent K_m. For competitive inhibitors at a substrate concentration significantly less than K_m, the $IC_{50}=K_i$ (9). Luminescence was recorded 30 and 60 minutes after reagent addition. GraphPad Prism® software was used to calculate the IC_{50} of 3.5nM.

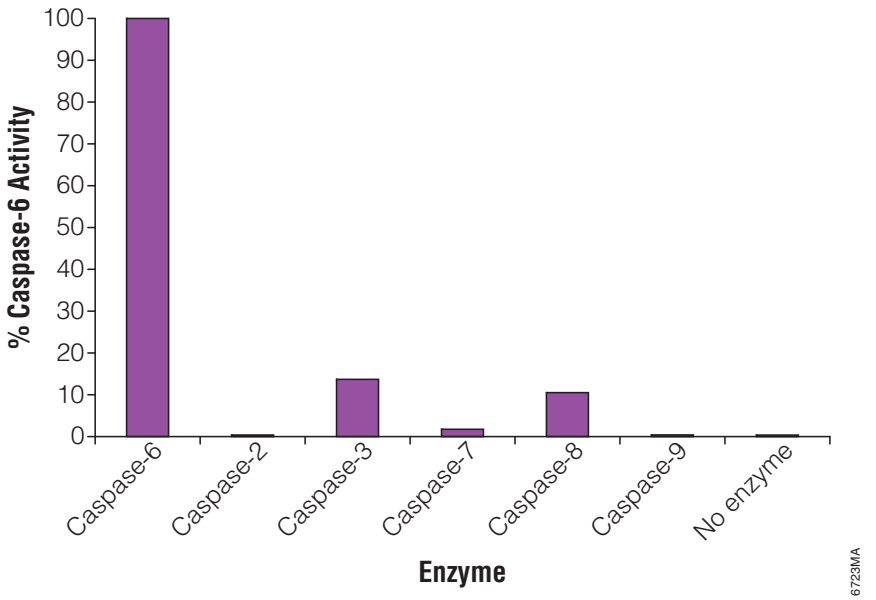

Figure 7. Cross-reactivity of the Caspase-Glo® 6 Assay with other caspases. The Caspase-Glo® 6 Assay was performed comparing human recombinant caspases-2, -3, -7, -8 and -9 (BIOMOL International) to caspase-6. The purified enzymes were serially diluted in HEPES buffer (10mM HEPES buffer [pH 7.2], 2mM DTT and 0.1% Prionex® as a carrier). Luminescence was recorded as relative light units (RLU) on a GloMax® 96 Microplate Luminometer 30 minutes after adding the Caspase-Glo® 6 Reagent. To normalize for units of caspase activity, the following protein concentrations (in reaction) were compared: 70pg/ml caspase-6; 7.5ng/ml caspase-2; 78pg/ml caspase-3; 110pg/ml caspase-7; 70pg/µl caspase-8; 7.7ng/µl caspase-9. Caspases-3, -7, and -8 generated activity above background. Caspases-2 and -9 resulted in no cross-reactivity even though 100X the amount of protein was added to the reaction.

2. Product Components and Storage Conditions

Product	Size	Cat.#
Caspase-Glo® 6 Assay	10ml	G0970

Cat.# G0970 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 Caspase-Glo® 6 Buffer
- 100µl Z-VEID-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Product	Size	Cat.#
Caspase-Glo® 6 Assay	50ml	G0971

Cat.# G0971 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 Caspase-Glo® 6 Buffer
- 500µl Z-VEID-Glo™ Substrate
- 1 bottle Luciferin Detection Reagent

Storage Conditions: Store the Caspase-Glo® 6 Assay components at –20°C protected from light. The Caspase-Glo® 6 Buffer and Z-VEID-Glo™ Substrate may be thawed and stored at 4°C for 2 months with no loss in signal. The Z-VEID-Glo™ Substrate also can be refrozen and stored at –20°C. Caspase-Glo® 6 Reagent (combined Z-VEID-Glo™ Substrate, Caspase-Glo® 6 Buffer and Luciferin Detection Reagent) can be refrozen and stored at –20°C for 1 week with no loss in signal. For best results, we recommend storing the reconstituted reagent in single-use aliquots at –20°C. The reagent can be thawed and frozen several times with minimal loss of signal.

3. Reagent Preparation and Storage

1. Thaw the Caspase-Glo® 6 Buffer, and equilibrate both the buffer and lyophilized Luciferin Detection Reagent to room temperature (22–25°C) before use.
2. Reconstitute the Luciferin Detection Reagent in the amber bottle by adding Caspase-Glo® 6 Buffer (10ml for Cat.# G0970, 50ml for Cat.# G0971). The Luciferin Detection Reagent should go into solution easily in less than 1 minute.
3. Thaw the Z-VEID-Glo™ Substrate, and mix well by vortexing briefly before use.

3. Reagent Preparation and Storage (continued)

4. Prepare the Caspase-Glo® 6 Reagent by adding the Z-VEID-Glo™ Substrate to the resuspended Luciferin Detection Reagent. For Cat.# G0970, add 100µl of Z-VEID-Glo™ Substrate to the 10ml of Luciferin Detection Reagent. For Cat.# G0971, add 500µl of the Z-VEID-Glo™ Substrate to the 50ml of Luciferin Detection Reagent. Mix by swirling or inverting the contents to obtain a homogeneous solution. The Z-VEID-Glo™ Substrate will be at 10µM concentration in the Caspase-Glo® 6 Reagent. The apparent K_m for the substrate is 5µM.

5. Allow the Caspase-Glo® 6 Reagent to incubate at room temperature for 30–60 minutes prior to use. This allows 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).

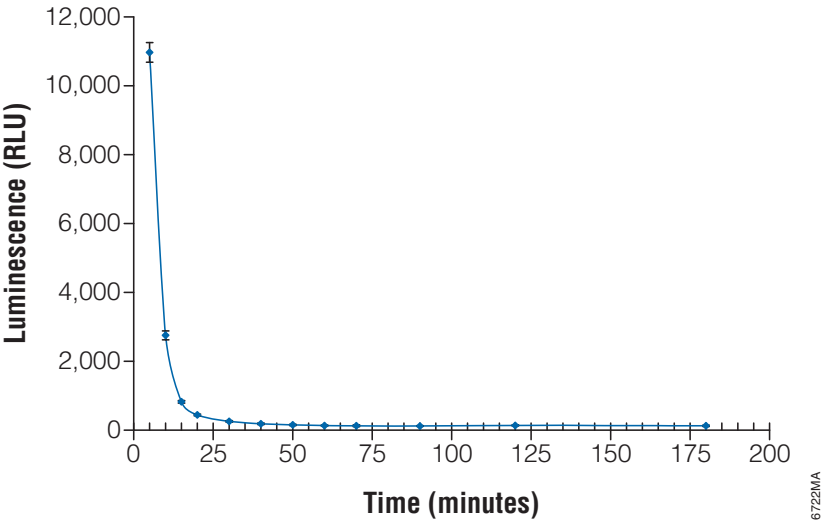

Figure 8. Time course for removal of free aminoluciferin from the Caspase-Glo® 6 Reagent. The Z-VEID-Glo™ Substrate was added to the bottle of reconstituted Luciferin Detection Reagent, and a time course of luminescence loss was recorded. Trace amounts of free aminoluciferin are present in the substrate and are removed by incubation with the reconstituted Luciferin Detection Reagent. To achieve maximal assay sensitivity with minimal background luminescence, the prepared Caspase-Glo® 6 Reagent should be incubated for at least 30 minutes at room temperature (22–25°C) before use.

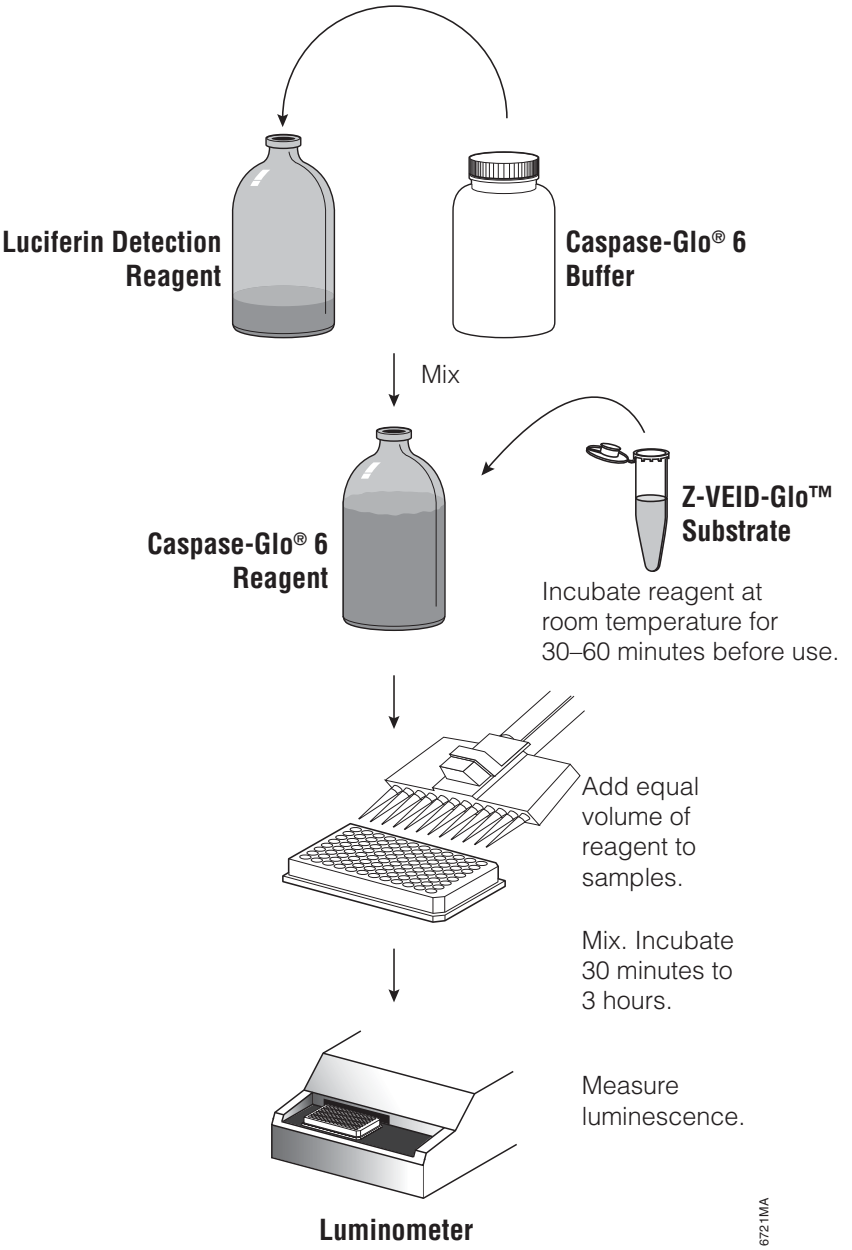

Figure 9. Schematic showing preparation and use of the Caspase-Glo® 6 Reagent.

4. Assay for Detection of Caspase-6 Activity

Directions are provided here for performing the Caspase-Glo® 6 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 Caspase-Glo® 6 Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl Caspase-Glo® 6 Reagent in a 384-well format).

Materials to Be Supplied by the User

- white multiwell plates (black plates may be used, but RLU will be reduced)
- multichannel pipette or automated pipetting station
- plate shaker or other device for mixing multiwell plates
- luminometer capable of reading multiwell plates
- caspase-6 enzyme (e.g., BIOMOL International Cat.# SE-170 or Calbiochem Cat.# 218799)
- optional: Prionex® carrier (Centerchem, Inc. Norwalk, CT.)

4.A. Assay Conditions

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

Blank: Caspase-Glo® 6 Reagent + vehicle control for test compound or inhibitor (no test compound or inhibitor included)

Positive Control: Caspase-Glo® 6 Reagent + vehicle control + purified caspase-6 enzyme

Assay: Caspase-Glo® 6 Reagent + treatment agent + purified caspase-6 enzyme

The blank is used as a measure of any background luminescence associated with the treatment agent vehicle and Caspase-Glo® 6 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 treatment agent used.

Notes:

1. Prepare the Caspase-Glo® 6 Reagent as described in Section 3, and mix thoroughly prior to starting the assay. Allow the prepared Reagent to sit at room temperature for at least 30 minutes to remove any contaminating free aminoluciferin.
2. The final concentration of caspase-6 enzyme should be 2U/ml or less (within the linear range of the assay, Figure 2).
3. The recommended caspase-6 dilution buffer is 10mM HEPES (pH 7.2), 2mM DTT and 0.1% Prionex® carrier (optional, use as a carrier if low enzyme concentrations are used).
4. Use identical enzyme concentrations for the assay and positive control reactions.
5. Gentle mixing may be performed using a plate shaker.
6. The maximal luminescent signal will be reached in ~30 minutes and will be stable for several hours (Figure 3).

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

1. Add 50µl of Caspase-Glo® 6 Reagent to each well of a white or black 96-well plate containing 50µl of blank, positive control or assay treatment.

   Notes:

   If reusing pipet tips, be careful not to touch pipet tips to the wells containing samples to avoid cross-contamination.

   Plates can be covered with a plate sealer if incubating for extended periods (>4 hours).

2. Gently mix the contents of the wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for 30 minutes to 3 hours (Figure 3, Panel A).

   Note: Maximal signal is typically reached within 30 minutes using caspase-6 enzyme (Figure 3, Panel B). At this time sensitivity is optimal. Temperature fluctuations will affect the luminescent readings; if the room temperature fluctuates significantly, a constant-temperature incubator may be used.

3. Record luminescence with a plate-reading luminometer.

5. General Considerations

Sensitivity

The bioluminescent Caspase-Glo® 6 Assay is more sensitive than comparable fluorescence assays for several reasons. Fluorescence substrates generally depend on a shift in the excitation/emission wavelengths after cleavage by the protease; consequently, there is some overlap in the emission spectra of the substrate before and after cleavage, creating substantial inherent background. The luminescent substrate (Z-VEID-aminoluciferin) is not a substrate for luciferase until it is cleaved; hence inherent background is insignificant. Furthermore, the homogeneous, coupled-enzyme format of the assay ensures that any contaminating free aminoluciferin is consumed before beginning the assay (Figure 8). In a fluorescence assay any contaminating free fluorophore remains, contributing to background. The only background in this bioluminescent assay results from the spontaneous hydrolysis of the Z-VEID-aminoluciferin substrate. Thus the inherent background is low, resulting in large signal-to-noise ratios (Figure 5). The low background also contributes to the broad range of linearity for the assay (3 logs of caspase-6 concentration; Figures 2 and 5). The assay sensitivity allows you to use less enzyme if screening for caspase-6 inhibitors. We recommend using ~2U/ml or less of caspase-6 per well (based on a BIOMOL International recombinant enzyme); at higher concentrations the kinetics of the assay are compromised due to product inhibition of the luciferase reaction.

5. General Considerations (continued)

Sensitivity (continued)

The assay is not dependent on accumulation of cleaved product because the light output is a result of the luciferase consuming aminoluciferin as soon as it is produced. Maximum signal and sensitivity are achieved as soon as the caspase-6 and luciferase activities reach a steady state. Typically this occurs in 30 minutes; therefore, the assay is extremely sensitive in a short time frame.

Temperature

The intensity and rate of decay of the luminescent signal from the Caspase-Glo® 6 Assay depends on the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction will result in a change in the intensity of light output and the stability of the luminescent signal. Temperature is one factor that affects the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature prior to performing the assay. For batch-mode processing of multiple assay plates, precautions should be taken to ensure complete temperature equilibration. Plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer for equilibration than plates arranged in a single layer.

Chemicals

The chemical environment of the luciferase reaction will affect the enzymatic rate and thus luminescence intensity. Solvents used for various chemical compounds may interfere with the luciferase reaction and thus 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 and found to have a minimal effect on light output.

Mixing

Mixing is not absolutely required after adding the Caspase-Glo® 6 Reagent, although it may aid in reproducibility between wells.

6. References

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7. Related Products

Luminescent Caspase Assays

Product	Size	Cat.#
Caspase-Glo® 2 Assay	10ml	G0940
Caspase-Glo® 3/7 Assay	2.5ml	G8090
Caspase-Glo® 8 Assay	2.5ml	G8200
Caspase-Glo® 9 Assay	2.5ml	G8210

Available in Additional Sizes.

Other Protease Assays

Product	Size	Cat.#
Proteasome-Glo™ Cell-Based Assay	10ml	G8660
Proteasome-Glo™ 3-Substrate System	10ml	G8531
Proteasome-Glo™ Chymotrypsin-Like Assay	10ml	G8621
Proteasome-Glo™ Trypsin-Like Assay	10ml	G8631
Proteasome-Glo™ Caspase-Like Assay	10ml	G8641
DPPIV-Glo™ Protease Assay	10ml	G8350
Calpain-Glo™ Protease Assay	10ml	G8501

Available in Additional Sizes.

7. Related Products (continued)

Cell Viability and Cytotoxicity Assays

Product	Size	Cat.#
CellTiter-Fluor™ Cell Viability Assay*	10ml	G6080
CellTiter-Glo® Luminescent Cell Viability Assay (ATP)*	10ml	G7570
MultiTox-Fluor Multiplex Cytotoxicity Assay*	10ml	G9200
CytoTox-Fluor™ Cytotoxicity Assay*	10ml	G9260
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay*	200 assays	G3582
CellTiter-Blue® Cell Viability Assay (resazurin)	20ml	G8080
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH)	200–800 assays	G7890

*Available in Additional Sizes.

[a]U.S. Pat. Nos. 7,148,030, 7,384,758 and 7,666,987, Japanese Pat. No. 4451663 and other patents pending.

[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.

[c]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.

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