DETECT CASPASE-8 AND -9 ACTIVITIES USING THE CASPASE-GLO™ ASSAYS

BY ANDREW NILES, M.S., KAY RASHKA, B.S., DEBORAH BISHOP, B.S., RICH MORAVEC, B.S., MARTHA O'BRIEN, PH.D., AND TERRY RISS, PH.D., PROMEGA CORPORATION

We have developed sensitive and homogeneous luminescent methods for measuring caspase-8 and -9 activities, which represent a marked improvement over existing fluorescent-based methods. The assay sensitivity is sufficient to detect caspase-8 or -9 activity from the number of cells typically used in a 96-well format. The assays are also efficient means to screen for specific inhibitors of the caspases in biochemical assays. These robust and homogeneous assays are ideal tools for studying the role of the caspase cascade in apoptosis.

Introduction

The dynamic flux within the cellular environment directs homeostasis in multicellular organisms. Cell death or survival depends upon a coordinated temporal and spatial program that is regulated by positive or negative stimuli. Although many of the parameters and factors governing these processes are still unknown, recent investigation has allowed insight into the complexities of programmed cell death by apoptosis.

The caspase family of cysteine proteases are the central mediators of the proteolytic cascade leading to cell death and elimination of compromised cells. As such, the caspases are tightly regulated both transcriptionally and by endogenous anti-apoptotic polypeptides, which block productive activation (1). Furthermore, the enzymes involved in this process dictate distinct pathways and demonstrate specialized functions consistent with their primary biological roles (2).

Pro-apoptotic stimuli can trigger caspase activation by two primary mechanisms. The extrinsic pathway involves receptor engagement or cross-linking of the tumor necrosis factor (TNF) superfamily of "death receptors," which promotes assembly of the death-inducing signaling complex (DISC) including the autocatalytic caspase-8 zymogen (3,4). The resulting active caspase-8 enzyme is capable of further processing and activating the

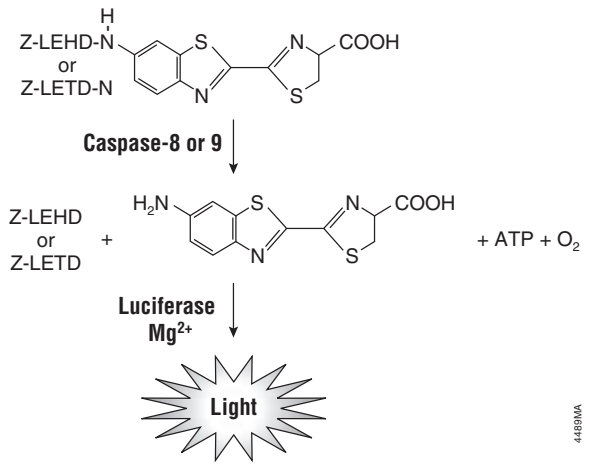

Figure 1. Caspase-8 or -9 cleavage of the proluminogenic substrates containing LETD or LEHD, respectively. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and production of light.

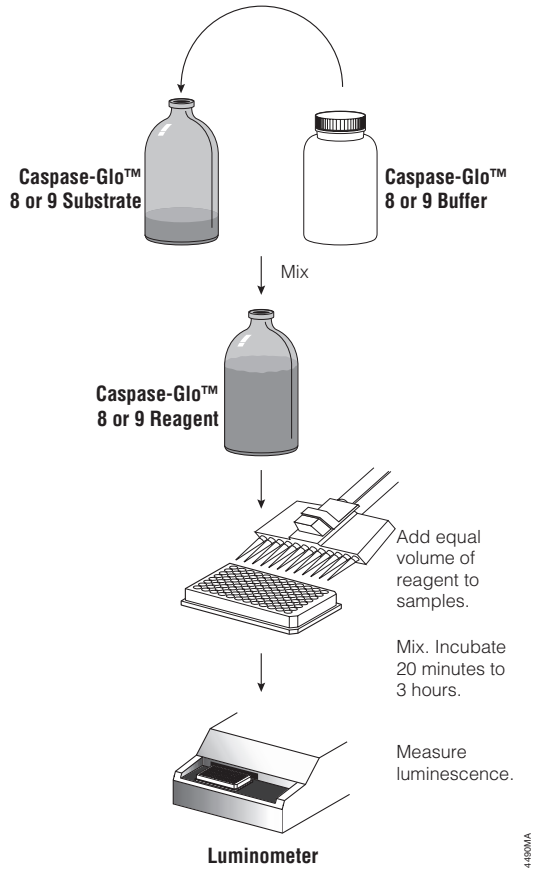

Figure 2. Schematic diagram of the Caspase-Glo™ Assay protocols.

"downstream" death-effector caspases (-3, -6, -7), leading to destruction of structural elements and repair enzymes (5).

The intrinsic or mitochondrial pathway is initiated by viral, ultraviolet (UV) or cell membrane insults leading to cytochrome c release into the cytosol. Through a complex sequence of events, procaspase-9, Apaf-1, dATP and cytochrome c assemble to a more catalytically active complex known as the "apoptosome" (6,7). As in the case of caspase-8, this apical caspase complex can lead to activation of the death-effector caspases that mediate apoptosis.

Further dissection of the caspase pathways are required to understand the inherent apoptotic checkpoints and their impact on health maintenance or disease. Currently available

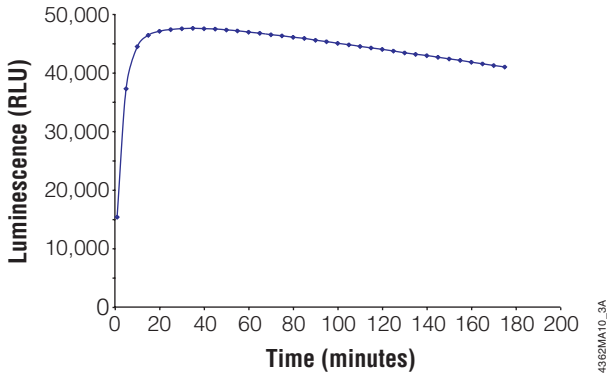

Figure 3. The Caspase-Glo™ Assays achieve optimal sensitivity within minutes and demonstrate a persistent "glow-type" luminescence. Purified recombinant caspase-8 enzyme (Biomol) was assayed in a total volume of 200µl per well in a solid white 96-well plate using the Caspase-Glo™ 8 Assay Reagent. Caspase-8 enzyme was diluted to 125U/ml in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer. One unit of caspase-8 is the amount of enzyme required to cleave 1pmol of substrate (Ac-LETD-pNA) per minute at 30°C per the manufacturer's unit definition. Each point represents the average of 4 wells. Unit definitions of caspase-8 activity may vary between manufacturers; the number of units may not translate directly between vendors.

methodologies generally lack the sensitivity for simple, efficient and cost-effective study of the caspase network. We describe an improved caspase assay for furthering this research.

Caspase-Glo™ Assay Principle

The Caspase-Glo™ Assays[a,b] use the luminogenic caspase-8 tetrapeptide substrate (Z-LETD-aminoluciferin) or caspase-9 tetrapeptide substrate (Z-LEHD-aminoluciferin) and a stable luciferase in proprietary buffers. The buffers are optimized for either caspase-8 or -9 activity, cell lysis and luciferase activity. In the absence of active caspase, the caspase substrates do not act as substrates for luciferase and thus produce no light. Upon cleavage of the substrates by the respective caspase, aminoluciferin is liberated and can contribute to the generation of light in a luminescence reaction (Figure 1). The resulting luminescent signal is directly proportional to the amount of caspase activity present in the sample.

Simple and Straightforward

The Caspase-Glo™ 8 and 9 Assays are configured for ease of operator use. The reagents are prepared by adding buffer directly to the lyophilized substrate. These homogeneous reagents can then be added to the sample in a convenient 1:1 ratio (Figure 2) without a separate lysis step. Because the luminescent signal "glows" rather than "flashes," reagent injectors are not required.

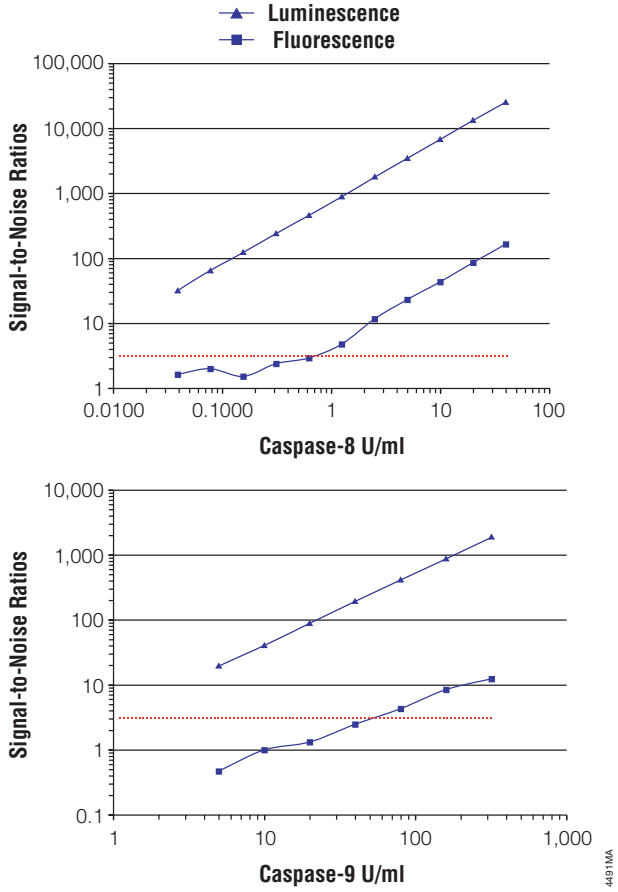

Figure 4. Caspase-Glo™ 8 and 9 Assays are more sensitive than comparable fluorescent assays. In these examples, recombinant caspase-8 or -9 was twofold serially diluted with 10mM HEPES (pH 7.6) with 0.1% Prionex® stabilizer. The diluted caspase-8 or -9 was mixed with an equal volume Capase-Glo™ 8 or 9 Reagent or respective AFC-labeled fluorescent substrate diluted in Caspase-Glo™ Buffer with 10mM DTT. Luminescence or fluorescence signal was measured at 1 hour on a BMG FLUOstar or Cytofluor® II, respectively. Triangle symbols represent Caspase-Glo™ Assay signal-to-noise ratios, whereas square symbols represent the S:N ratios derived from their fluorescent counterparts. The dotted line denotes a S:N ratio of 3, indicating a limit of detection equal to 3 standard deviations above background. For these caspase titrations, the Caspase-Glo™ Assays have not reached their limits of detection. The lowest doses are well below the limit of detection for the fluorescent assays. S:N ratio = [(average signal with enzyme) – (average signal of no-enzyme control)] / [standard deviation of no-enzyme control].

Faster Results

An optimum signal is achieved for the Caspase-Glo™ Assays within minutes after adding reagents to the samples (Figure 3). A steady state is achieved rapidly between the caspase and luciferase reactions that results in a luminescent signal lasting for hours (Figure 3) and provides flexibility for recording data. The time-to-result is much shorter compared to fluorescent assays that require extended incubations to achieve a

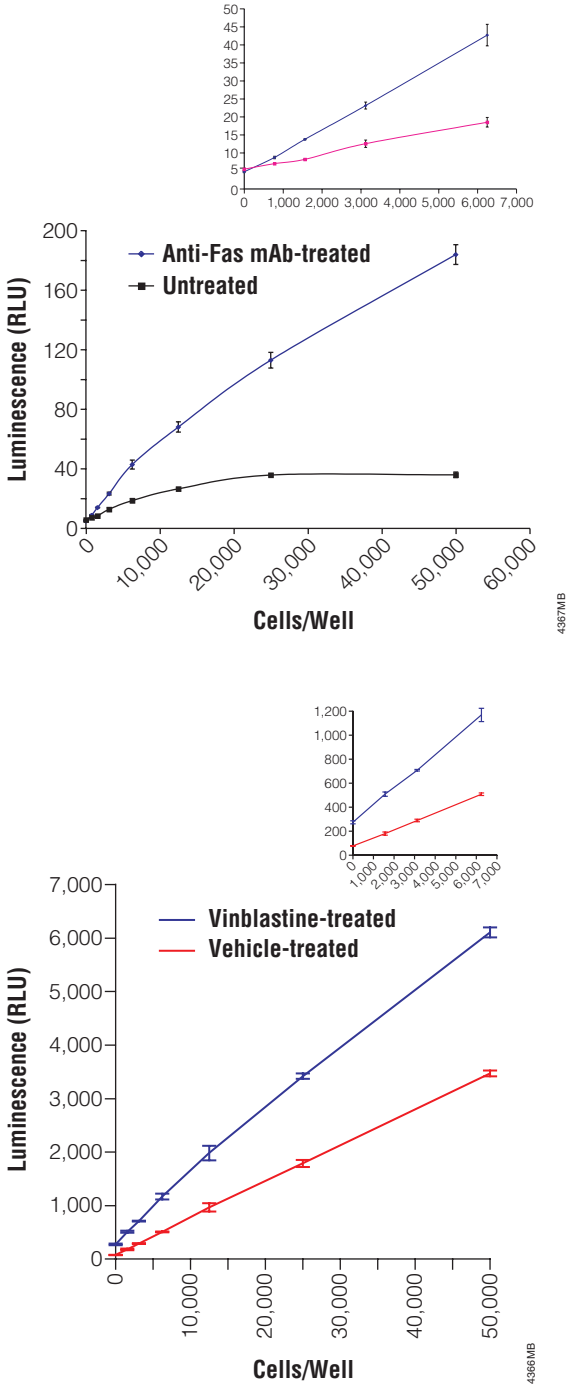

Figure 5. The Caspase-Glo™ Assays allow detection of apical caspase activities in multiwell formats. Serial dilutions of either Jurkat or HL-60 cells were prepared in clear-bottom 96-well plates in RPMI 1640 with 10% FBS. Jurkat cells were treated with Anti-Fas mAb (100ng/ml final) for 3 hours. HL-60 cells were treated with vinblastine (50µM final) for 3 hours. A vehicle diluent in medium served as control. Either Caspase-Glo™ 8 or 9 reagent was added directly to the wells, the plates mixed briefly by orbital shaking, incubated at room temperature, and luminescence measured at 30 minutes using a BMG FLUOstar and Dynex MLX® luminometer, respectively. Each point represents the mean ± S.D. of 3 wells.

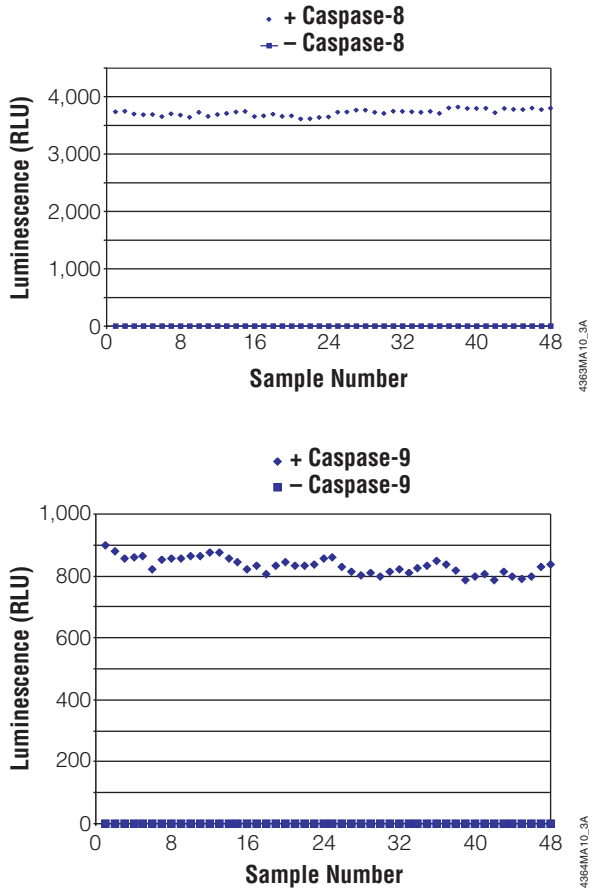

Figure 6. The Caspase-Glo™ Assays are suitable for HTS applications. Z´ factors (8) were calculated using recombinant caspase-8 or -9 (Biomol) at 10U/ml and with no-caspase buffer blanks. Assays were performed in a total volume of 200µl in a white 96-well plate (48 wells/sample and control). Half of the wells of each plate contained buffer and purified caspase-8 or -9 (+ caspase-8 or -9), and half of the wells contained buffer only with no purified caspase (–caspase-8 or -9). Z´ factors were 0.957 and 0.903, respectively for the assays.

significant signal and suffer from high intrinsic background fluorescence that limits S:N ratios.

Improved Sensitivity

The Caspase-Glo™ Assays produce a linear response with caspase concentration and have signal windows that are several orders of magnitude greater than assays using fluorescence-based substrates (Figure 4). This improved sensitivity allows for homogeneous detection of caspase activities in cell-based assays in multiwell plates with standard concentrations of cells (Figure 5). Conversely, fluorescence assays typically require cell washing, concentration and preparation of lysates to achieve detectable concentrations of active caspase.

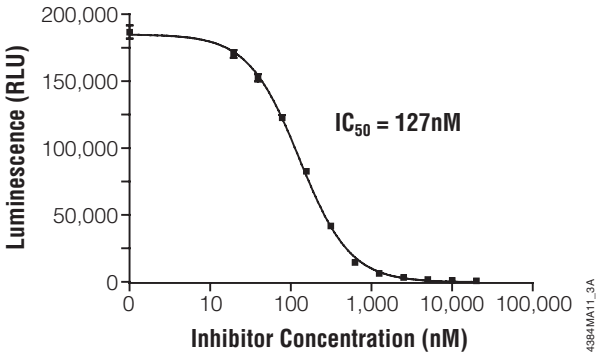

Figure 7. Inhibition potencies can be quickly and reliably determined using the Caspase-Glo™ Assays. The reversible caspase inhibitor Ac-LETD-CHO (Sigma) was twofold serially diluted in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex® stabilizer and added to a solid white 96-well plate in 50µl volumes. Recombinant caspase-8 (Biomol) at 10U/ml was added in an equal volume to the plate, mixed by orbital shaking and allowed to reach inhibition equilibrium (30 minutes). Caspase-Glo™ 8 Reagent was added (100µl), and luminescence was measured at 1 hour on a BMG FLUOstar luminometer. Data were graphed using GraphPad Prism® software and fitted by nonlinear regression for determination of the IC_{50} value for the compound.

HTS Applications

The Caspase-Glo™ Assays are suited for high-throughput screening because of their high sensitivity and excellent Z´-factor values (Figure 6; ref. 8). These single-addition assays can accommodate scaled volumes and reduce the amount of enzyme or test compound used, leading to a savings in screening costs. Furthermore, both assays have been validated for use by liquid-handling automated workstations.

Conventional Kinetic Applications

Typically, researchers need to define the potency of a compound or treatment for the induction or inhibition of caspases. The Caspase-Glo™ 8 and 9 Assays allow convenient and efficient definition of kinetic constants and provide useful information for drug selection or prioritization (Figure 7). The ED_{50} or IC_{50} values can be determined quickly at luminescence steady state.

Summary

Because of the differential means of activating or blocking an apoptotic program, identifying the factors leading to the apoptotic defects in disease models has therapeutic relevance. The homogeneous nature, sensitivity and simplicity of use of the Caspase-Glo™ Assays allow careful dissection of this important homeostatic mechanism. When used in conjunction with other established methodologies (e.g., Western blotting, immunochemical labeling, RNA interference, etc.), measuring caspase activities with the Caspase-Glo™ Assays can reveal valuable information regarding the mechanism of the apoptotic process. ■

References

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Protocols

Caspase-Glo™ 8 Assay Technical Bulletin #TB332
(www.promega.com/tbs/tb332/tb332.html)

Caspase-Glo™ 9 Assay Technical Bulletin #TB333
(www.promega.com/tbs/tb333/tb333.html)

Automated Caspase-Glo™ Assays Protocol #EP017
(www.promega.com/tbs/ep017/ep017.html)

Ordering Information

Product	Size	Cat.#
Caspase-Glo™ 8 Assay[a,b]	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo™ 9 Assay[a,b]	2.5ml	G8210
	10ml	G8211
	100ml	G8212

For Laboratory Use.

[a] The method of recombinant expression of Coleoptera luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

[b] U.S. Pat. No. 6,602,677 and Australian Pat. No. 754312 have been issued to Promega Corporation for thermostable luciferases and methods of production. Other patents are pending.