

CASPASE-GLO™ 3/7 ASSAY: USE FEWER CELLS AND SPEND LESS TIME WITH THIS HOMOGENEOUS ASSAY

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The newly developed Caspase-Glo™ 3/7 Assay allows researchers to detect caspase-3 and -7 activity in as few as 20 anti-Fas mAb-treated Jurkat cells. This luminescent assay is designed for multiwell-plate formats, and because it requires no washing or removal of cell culture medium, it is ideal for high-throughput screening applications. The assay demonstrates excellent Z'-factor values, indicating a robust assay.

Introduction

Assay Principle and Protocol

Activation of the caspase cascade is an integral event in the apoptotic pathway. Caspase-3 is one of the important "executioner" caspase enzymes, triggering the cleavage of numerous proteins that lead to the ordered breakdown of the cell. The detection of caspase-3 activation is frequently used as a positive marker for apoptosis. We have developed a homogeneous luminescent caspase-3/7 assay, the Caspase-Glo™ 3/7 Assay^(a) that provides an extremely sensitive means of monitoring caspase activation in both cells and in purified enzyme preparations. The assay uses a proluminescent substrate containing the DEVD sequence recognized by caspase-3 and -7.

The assay provides the proluminescent caspase-3/7 substrate and a thermostable luciferase in a buffer system optimized for caspase-3/7 activity, luciferase activity, and cell lysis. Adding a single Caspase-Glo™ 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate, liberating free aminoluciferin that is consumed by luciferase, and generating a "glow-type" luminescent signal (Figure 1). The luminescent signal produced is proportional to the amount of caspase activity present. This homogeneous Caspase-Glo™ 3/7 Assay is designed for use with multiwell-plate formats, making it ideal for automated high-throughput

screening (HTS) of caspase activity or apoptosis. Cell washing, removal of medium and multiple pipetting steps are not required.

Luminescence is Proportional to Caspase-3 Activity

The Caspase-Glo™ 3/7 Reagent produces luminescence that is directly proportional to the amount of caspase-3/7 activity. The luminescence is linear over four orders of magnitude of caspase concentration (Figure 2) and over a broad range of cell densities (Figure 3). The luminescent signal is also stable with little decrease in signal intensity over four hours (Figure 4). This stability allows use of luminometers without reagent injectors, and luminescence measurements can be delayed for several hours.

Achieve Maximum Signal in Minimal Time

Caspase assays that use fluorescent substrates depend on the accumulation of released fluorophore. Consequently, the signal-to-background ratio increases over time as free fluorophore accumulates following caspase cleavage, and sensitivity can be increased by longer incubations. In contrast, this homogeneous, luminescent assay is not dependent on the accumulation of caspase-cleaved product. The caspase cleavage of the proluminescent substrate results in the release of aminoluciferin; aminoluciferin is then immediately consumed by luciferase. Maximum sensitivity is achieved once the caspase and luciferase activities reach steady state. Steady state, and thus maximum signal, is reached in approximately 1 hour. The precise time to reach maximum signal will depend on the assay system and culturing conditions. Compared to fluorescent assays, this luminescent assay achieves greater sensitivity in a shorter time, and the signal remains quite stable for several hours.

A Highly Sensitive Assay Requires Fewer Cells

The sensitivity of fluorescent assays can be limited because of fluorescence background derived from three possible sources: 1) the spectra of the peptide-conjugated and unconjugated fluorophore may overlap slightly, causing inherent fluorescence background; 2) incomplete purification of peptide-conjugated fluorophore, resulting in fluorescence from contaminating free fluorophore; and 3) autofluorescence from the cells or compounds being assayed.

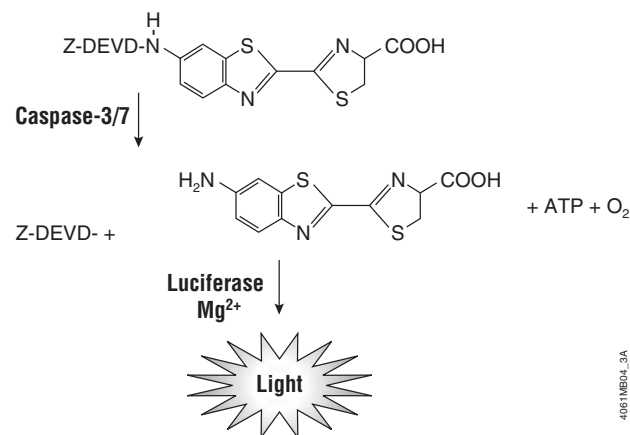


Figure 1. The Caspase-Glo™ reaction.

Caspase-Glo™ 3/7 Assay

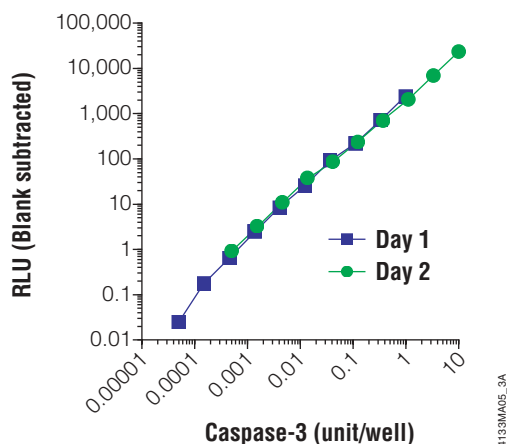


Figure 2. The Caspase-Glo™ 3/7 Assay is linear over four orders of magnitude of caspase concentration. Purified caspase-3 was titrated and assayed in 96-well plates using the Caspase-Glo™ 3/7 Assay on two different days. Luminescence was measured 1 hour after adding the Caspase-Glo™ Reagent to the cells. The graph shows that the assay is linear over 4 orders of magnitude of caspase concentration. One unit of caspase = 0.07ng protein = 1pmol of substrate (Ac-DEVD-pNA) hydrolyzed/minute per the manufacturer's unit definition. Each point represents the average of 4 wells. The no-caspase blank control was subtracted from each point.

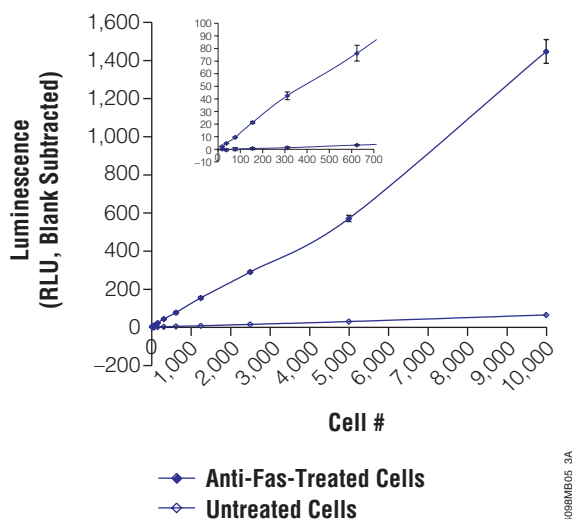


Figure 3. The Caspase-Glo™ 3/7 Assay produces luminescence that is linear over a broad range of cell numbers. Jurkat cells either were treated with anti-Fas mAb for 4.5 hours to induce apoptosis or were left untreated. Caspase-Glo™ 3/7 Reagent was added directly to the cells in 96-well plates and incubated for 1 hour before recording luminescence. Each point represents the average of 4 wells. The "no cell" blank control value has been subtracted from each. The assay is linear over a broad range of cell number.

In the luminescent Caspase-Glo™ 3/7 Assay, the peptide-conjugated aminoluciferin is not a suitable substrate for luciferase, so there is virtually no inherent luminescence; any contaminating free aminoluciferin is consumed by the luciferase before the assay is initiated, and autoluminescence

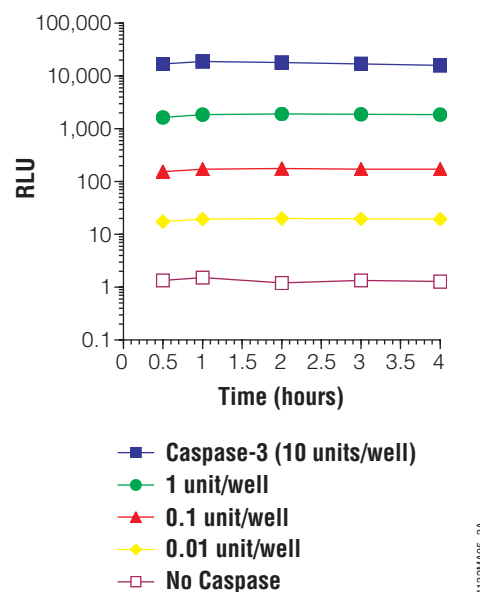


Figure 4. The Caspase-Glo™ Assay luminescent signal is stable for several hours. Purified caspase-3 enzyme was titrated and assayed in 96-well plates using the Caspase-Glo™ 3/7 Assay. The assay gives a luminescent signal that is stable for several hours. One unit caspase = 0.1ng protein = 1pmol of substrate (Ac-DEVD-pNA) hydrolyzed/minute.

is not a significant problem. The background luminescence in this assay is very low, resulting in excellent signal-to-background ratios and sensitivity not achieved by other caspase assay formats (1–3). The assay can detect as little as 0.1pg of active caspase-3 (Figure 2). In a titration of Jurkat cells induced to undergo apoptosis with anti-Fas mAb, caspase activity was detected in as few as 20 anti-Fas mAb-treated cells (Figure 3). Because of the sensitivity of this assay, fewer cells/well are required for accurate caspase activity measurements, and less recombinant enzyme is required if performing inhibitor screens.

Z'-Factor Analysis Indicates a Robust Assay

The assay demonstrates an excellent Z'-factor, a statistical value that compares the dynamic range of an assay to data variation in order to assess assay quality. Z'-factors between 0.5–1.0 indicate excellent assay quality (4). We calculated Z'-factor using recombinant caspase-3 (Figure 5) and obtained a Z'-factor value of 0.92, indicating a robust assay.

Applications: Determining EC₅₀ and IC₅₀ Values

The sensitivity and simplicity of this assay make it ideally suited for high-throughput screening. When apoptosis activators or caspase inhibitors are identified, the EC₅₀ and IC₅₀ values can be readily calculated using this assay. Figure 7 illustrates a typical EC₅₀ experiment using the apoptosis-inducing compound, staurosporine, on HeLa cells. The EC₅₀ value obtained with the Caspase-Glo™ 3/7 Assay of

Caspase-Glo™ 3/7 Assay

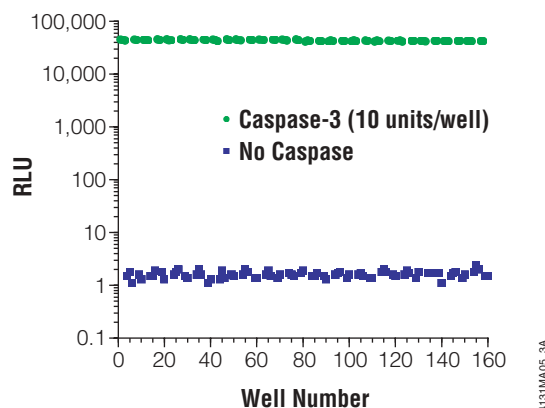


Figure 5. The Caspase-Glo™ 3/7 Assay can produce excellent Z'-factor values. A Z'-factor value was calculated using recombinant caspase-3 (10 units/well) and a no-caspase control in two 96-well plates (80 wells/plate). Half of the wells of each plate were + caspase, and half were – caspase (controls). Z'-factor = 0.92 for this assay. 1 unit caspase = 0.1ng protein = 1pmol of substrate (Ac-DEVD-pNA) hydrolyzed/minute, as per the manufacturer's unit definition.

200nM is comparable to the published EC₅₀ value of 100nM for staurosporine (5) in neuroblastoma cells. Figure 7 illustrates an experiment to determine the IC₅₀ value for the caspase-3 inhibitor, Ac-DEVD-CHO, using the Caspase-Glo™ Assay. The IC₅₀ for Ac-DEVD-CHO in this experiment is 330pM. The published K_i for this inhibitor is 230pM (6).

Conclusion

The Caspase-Glo™ 3/7 Assay provides a sensitive means of determining caspase-3 and -7 activity in cells or in purified enzyme extracts. The simplified protocol for the assay (add-mix-measure) and its design for a multiwell format make it ideal for high-throughput screening. Additionally the assay can be used to determine characteristic values such as EC₅₀ or IC₅₀ for caspase-3/7 activators or inhibitors, and the assay can produce excellent Z'-factor values indicating its utility as a robust screening tool. The stable signal allows for batch processing of large numbers of samples as well. ■

References

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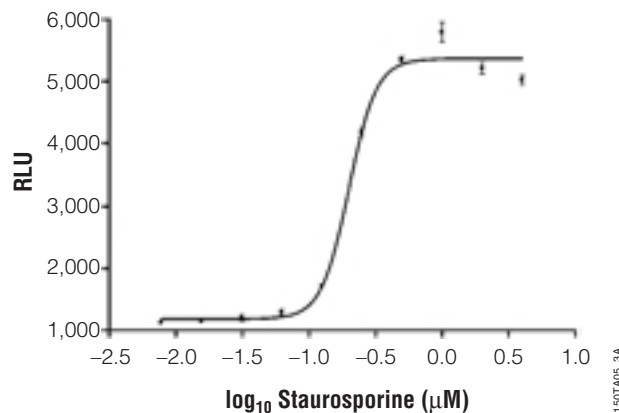


Figure 6. The Caspase-Glo™ 3/7 Assay can be used to obtain EC₅₀ values. HeLa cells in 96-well plates (10,000 cells/well) were treated with staurosporine for 4.5 hours. Caspase-Glo™ Reagent was added to each well, and readings were taken on a luminometer 1 hour after adding reagent. Data were graphed, and an EC₅₀ value was obtained using GraphPad Prism® software.

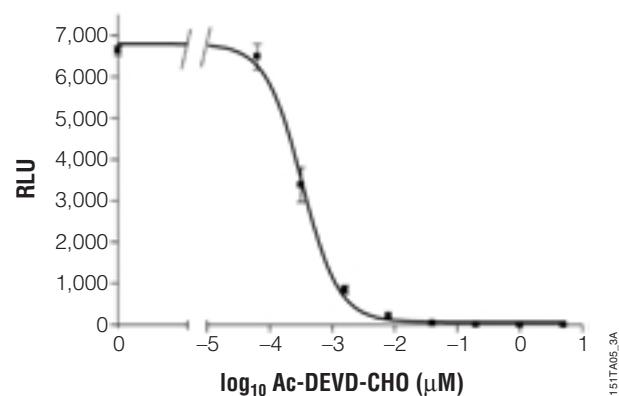


Figure 7. The Caspase-Glo™ Assay can be used to obtain IC₅₀ values. A titration of the caspase-3 inhibitor, Ac-DEVD-CHO, was combined with caspase-3 (1 unit/well) in 96-well plates. The Caspase-Glo™ Reagent was added to each well, and readings were taken on a luminometer 1 hour after adding reagent. Data were graphed and an IC₅₀ value was obtained using GraphPad Prism® software.

Protocol

Caspase-Glo™ 3/7 Assay Technical Bulletin #TB323
(www.promega.com/tbs/tb323/tb323.html)

Ordering Information

Product	Size	Cat.#
Caspase-Glo™ 3/7 Assay ^(a)	2.5ml	G8090
	10ml	G8091
	100ml	G8092

^(a)Patent Pending.

Caspase-Glo is a trademark of Promega Corporation.

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