

TECHNICAL MANUAL

Caspase-Glo[®] 1 Inflammasome Assay

Instructions for Use of Products
G9951, G9952 and G9953.



Caspase-Glo[®] 1 Inflammasome Assay

All technical literature is available at: www.promega.com/protocols/
 Visit the web site to verify that you are using the most current version of this Technical Manual.
 E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

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1. Description

The Caspase-Glo[®] 1 Inflammasome Assay^(a,b) is a homogeneous, bioluminescent method to selectively measure the activity of caspase-1, a member of the cysteine aspartic acid-specific protease (caspase) family and an essential component of the inflammasome (1). Inflammasomes are protein complexes induced by diverse inflammatory stimuli. Innate immune cells respond to pathogens and other danger signals with inflammasome formation and conversion of procaspase-1 zymogen into catalytically active caspase-1. Caspase-1 activation results in: 1) the processing and release of cytokines IL-1 β and IL-18; and 2) pyroptosis, an immunogenic form of cell death (2–5).

1. Description (continued)

The Caspase-Glo® 1 Inflammasome Assay provides a luminogenic caspase-1 substrate, Z-WEHD-aminoluciferin, in a lytic reagent optimized for caspase-1 activity and luciferase activity. A single addition of this reagent results in cell lysis, substrate cleavage by caspase-1 and generation of light by a proprietary, thermostable, recombinant luciferase (Ultra-Glo™ Recombinant Luciferase) as described in Figure 1. The coupled-enzyme system reaches a steady state between caspase cleavage of the substrate and luciferase conversion of aminoluciferin. These simultaneous reactions generate a stable luminescent signal, which is proportional to caspase activity (Figure 2). Inclusion of the proteasome inhibitor, MG-132, in the reagent eliminates nonspecific proteasome-mediated cleavage of the substrate, enabling sensitive detection of caspase-1 activity.

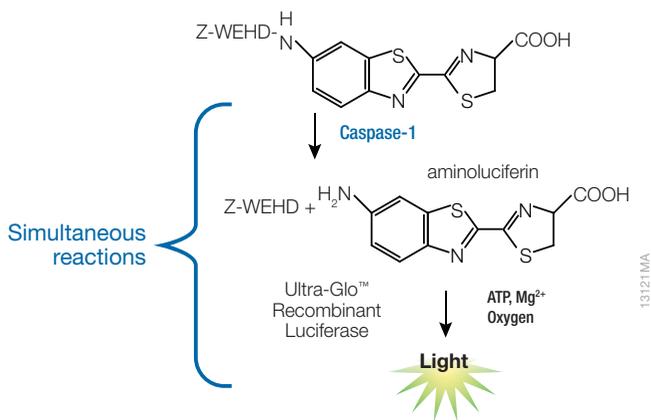


Figure 1. Caspase-Glo® 1 Inflammasome Assay chemistry. Following caspase cleavage of the Z-WEHD Substrate (i.e., Z-WEHD-aminoluciferin), a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and light production.

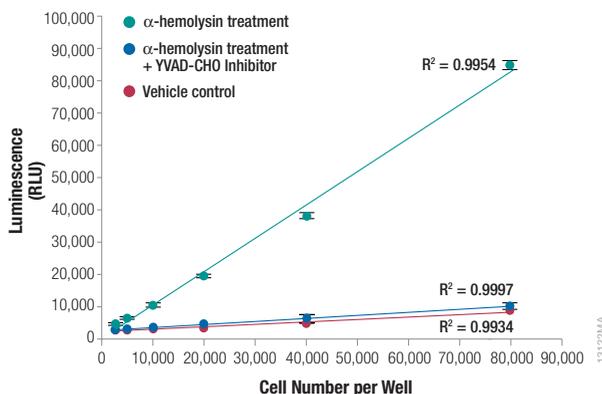


Figure 2. Caspase-Glo® 1 Inflammasome Assay is linear. THP-1 cells grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂ were titrated in 96-well plates and treated with α-hemolysin (2.0µg/ml, 2.5 hours) before adding Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent. Luminescence was recorded on a GloMax® Multi+ Detection System.

The Caspase-Glo® 1 Inflammasome Assay is designed for use with multiwell plates in both 96-well and 384-well formats, making the assay ideal for automated high-throughput screening of caspase activity or inflammasome activation. Cell washing, medium removal and multiple pipetting steps are not required (Figure 3). This novel caspase-1 assay system allows a more efficient and effective assessment of inflammasome activation and enables high-throughput screening for inflammasome modulators.

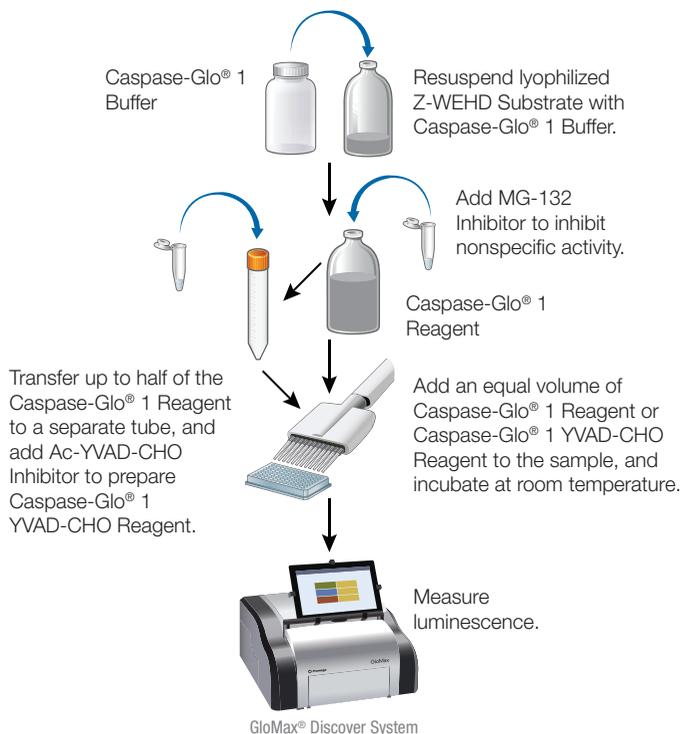


Figure 3. Schematic diagram of the Caspase-Glo® 1 Inflammasome Assay cell-based protocols.

1. Description (continued)

To verify caspase-1 specificity, the Caspase-Glo® 1 Assay includes a caspase-1 selective inhibitor, Ac-YVAD-CHO (6). This inhibitor inhibits 99% of caspase-1 activity but does not substantially inhibit any of the cross-reacting caspases (Figure 4). Performing the assay in parallel wells with and without Ac-YVAD-CHO enables you to measure caspase-1 activity specifically. For more information about assay specificity, see Section 6.B.

The Caspase-Glo® 1 Assay can be used to measure caspase-1 activity directly in cell cultures or released caspase-1 activity in culture medium. Monitoring released caspase-1 activity from culture medium is nondestructive, enabling use of the biological sample with other assays. The Caspase-Glo® 1 Assay also can be used to monitor caspase-1 activation in response to a wide variety of inflammasome inducers. Representative data are provided in Section 5.

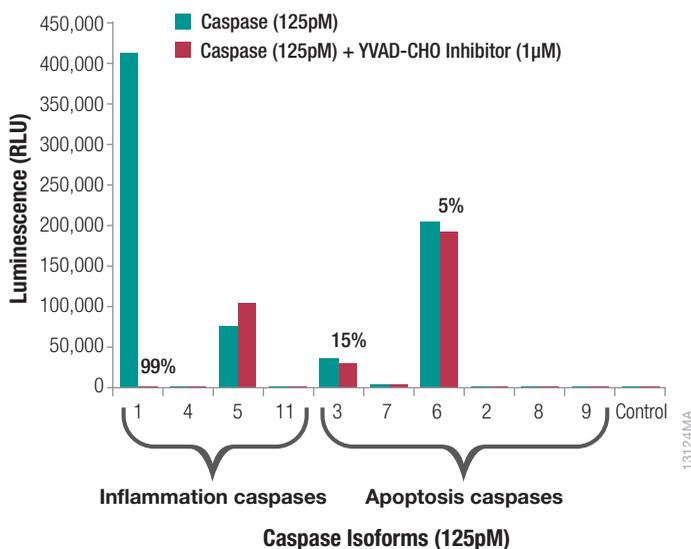


Figure 4. Z-WEHD-aminoluciferin substrate and Ac-YVAD-CHO Inhibitor selectivity. Ten caspases were assayed at 125pM with and without Ac-YVAD-CHO Inhibitor at a final concentration of 1µM. Caspase-11 was the only mouse caspase; all others were human. Caspase-8 is also a noncanonical inflammation caspase. Percentages indicate the amount of inhibition with Ac-YVAD-CHO Inhibitor.

Advantages of the Caspase-Glo® 1 Inflammasome Assay include:

Simple Method: The assay measures caspase-1 activity directly in cells or in medium from cultured cells in multiwell plates without lysate preparation or multiple pipetting steps. The homogeneous protocol makes the assay amenable to automation in 96- and 384-well formats.

Specificity for Caspase-1: The selective caspase-1 substrate, Z-WEHD-aminoluciferin, and MG-132 Inhibitor in the Caspase-Glo® 1 Reagent enable direct detection of caspase-1 activity in cells or culture medium in multiwell plates. Addition of the Caspase-Glo® 1 YVAD-CHO Reagent to parallel wells confirms the activity is caspase-1 specific (see Section 6.B).

Speed: No sample preparation or manipulation is required. The Caspase-Glo® 1 Reagent is added to wells and luminescence measured after only 1 hour for cells and cell culture medium. Less time and labor are required compared to Western blot analysis and ELISA.

Accuracy: The Caspase-Glo® 1 Assay measures only catalytically active caspase-1, enabling precise time courses of enzyme function (see Section 6.A). Western blots and ELISAs do not necessarily monitor active enzyme.

Greater Sensitivity: The Caspase-Glo® 1 Assay provides the sensitivity required to measure caspase-1 activity (see Section 6.B).

Flexible Plate Setup: An equal volume of reagent is added to cell culture medium in sample wells, enabling easy scaling to different multiwell plate formats.

Batch Processing: The luminescent caspase-1 signal is stable in the Caspase-Glo® 1 Reagent (half-life >3 hours), allowing plates to be read over a few hours. There is no need to use a luminometer with reagent injectors.

Assay Multiplexing: Caspase-1 activity can be monitored in culture medium, preserving the biological sample for use with other assays. In addition, same-well multiplexing can be performed with compatible assay chemistries (e.g., CellTox™ Green Cytotoxicity Assay).



2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Caspase-Glo® 1 Assay	10ml	G9951

The system contains sufficient reagents for 100 assays at 100µl per assay in a 96-well plate or 400 assays of 25µl per assay in a 384-well plate. Includes:

- 1 × 10ml Caspase-Glo® 1 Buffer
- 1 bottle Z-WEHD Substrate (lyophilized)
- 2 × 30µl MG-132 Inhibitor
- 1 × 10µl Ac-YVAD-CHO Inhibitor

PRODUCT	SIZE	CAT.#
Caspase-Glo® 1 Assay	5 × 10ml	G9952

The system contains sufficient reagents for 500 assays at 100µl per assay in a 96-well plate or 2,000 assays of 25µl per assay in a 384-well plate. Includes:

- 5 × 10ml Caspase-Glo® 1 Buffer
- 5 bottles Z-WEHD Substrate (lyophilized)
- 1 × 300µl MG-132 Inhibitor
- 5 × 10µl Ac-YVAD-CHO Inhibitor

PRODUCT	SIZE	CAT.#
Caspase-Glo® 1 Assay	100ml	G9953

The system contains sufficient reagents for 1,000 assays at 100µl per assay in a 96-well plate or 4,000 assays of 25µl per assay in a 384-well plate. Includes:

- 1 × 100ml Caspase-Glo® 1 Buffer
- 1 bottle Z-WEHD Substrate (lyophilized)
- 2 × 300µl MG-132 Inhibitor
- 1 × 100µl Ac-YVAD-CHO Inhibitor

Storage Conditions: Store the Caspase-Glo® 1 Buffer, Z-WEHD Substrate, MG-132 Inhibitor and Ac-YVAD-CHO Inhibitor at –20°C protected from light. The Caspase-Glo® 1 Buffer may be thawed and stored at 4°C for up to 3 months or at room temperature for up to 4 days with no loss in signal. See the product label for expiration date.

3. Reagent Preparation and Storage

Prepare the Caspase-Glo® 1 Reagent and Caspase-Glo® 1 YVAD-CHO Reagent on the day that the reagents will be used. We do not recommend storing these reagents for more than one day. If you will not use the entire volume of prepared reagents in one day, we recommend reconstituting and storing the Z-WEHD Substrate as described below and adding the MG-132 Inhibitor and Ac-YVAD-CHO Inhibitor on the day that the Caspase-Glo® 1 Reagent and Caspase-Glo® 1 YVAD-CHO Reagent will be used. If you have already confirmed the specificity of the model system, use of the Caspase-Glo® 1 YVAD-CHO Reagent may not be necessary. Adjust the quantity of prepared reagents to meet your needs.

1. Equilibrate the Caspase-Glo® 1 Buffer and Z-WEHD Substrate to room temperature before use.
2. Transfer the contents of one Caspase-Glo® 1 Buffer bottle to one amber bottle containing the Z-WEHD Substrate to reconstitute the Z-WEHD Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved. After reconstitution, the Z-WEHD-aminoluciferin concentration is 40µM, yielding a final substrate concentration in the assay of 20µM, the apparent K_m value for caspase-1.

3. Thaw the MG-132 Inhibitor and Ac-YVAD-CHO Inhibitor at room temperature.

Note: These inhibitors are not necessary for a biochemical enzyme assay. The reagent is ready for use if performing an assay with recombinant caspase-1. See Section 4.C.

4. Add 60µl of MG-132 Inhibitor to 10ml (or 600µl of MG-132 to 100ml) of reconstituted Z-WEHD Substrate to prepare the Caspase-Glo® 1 Reagent. Mix by swirling or inverting the contents until the reagent is thoroughly mixed. The MG-132 Inhibitor concentration in the Caspase-Glo® 1 Reagent is 120µM, yielding a final concentration of 60µM in the assay.

Note: If you are using the 10ml kit (Cat.# G9951), you can add both tubes of MG-132 Inhibitor to the entire 10ml bottle of reconstituted Z-WEHD Substrate. Alternatively, you can add one tube (30µl) of MG-132 Inhibitor to half of the reconstituted Z-WEHD Substrate (5ml). If you are using the 5 × 10ml system (Cat.# G9952), then add 60µl of MG-132 Inhibitor to each 10ml aliquot of reconstituted Z-WEHD Substrate. The MG-132 Inhibitor is stable for five additional freeze-thaw cycles. If you are using the 100ml kit (Cat.# G9953), add both tubes of MG-132 to the entire 100ml bottle of reconstituted Z-WEHD Substrate, or you can add one tube of MG-132 (300µl) to 50ml of the reconstituted Z-WEHD-Substrate.

5. Transfer up to half of the Caspase-Glo® 1 Reagent to a separate tube, and add the Ac-YVAD-CHO Inhibitor at a ratio of 1:1,000 (i.e., 5µl of Ac-YVAD-CHO Inhibitor to 5ml Caspase-Glo® 1 Reagent) to prepare the Caspase-Glo® 1 YVAD-CHO Reagent. Mix thoroughly by swirling or inverting. The Ac-YVAD-CHO concentration in the reagent is 2µM, and the final concentration in the assay is 1µM.

Note: If you have already confirmed the specificity of the model system, use of the Caspase-Glo® 1 YVAD-CHO Reagent may not be necessary. Adjust the quantity of prepared reagents to meet your needs.

Reagent Storage: Reconstituted Z-WEHD Substrate that is stored at 4°C for up to 2 days exhibits no loss of activity compared to freshly prepared reagent. Reconstituted Z-WEHD Substrate stored at 4°C for 1 week will give a signal approximately 80% of that obtained with freshly prepared reagent. Reconstituted Z-WEHD Substrate stored at -20°C for 1 week will give a signal approximately 85% of that of freshly prepared reagent, and reconstituted Z-WEHD Substrate stored at -20°C for 6 weeks will give a signal approximately 80% of that of freshly prepared reagent. The Caspase-Glo® 1 Reagent containing MG-132 Inhibitor can be stored overnight at 4°C or room temperature with no loss of activity. The MG-132 Inhibitor is stable for five additional freeze-thaw cycles but may be sensitive to prolonged exposure to light. The Ac-YVAD-CHO Inhibitor is stable for five additional freeze-thaw cycles.



4. Caspase-Glo® 1 Inflammasome Assay Protocols

Prior to performing the Caspase-Glo® 1 Assay, we recommend that you determine the optimal cell number and treatment period for your particular cells. As a general starting point, we recommend 40,000–60,000 cells per well in a 96-well plate.

4.A. Protocol for Measuring Caspase-1 Activity in Cultured Cells

This protocol describes assay setup in a total volume of 200µl using 96-well plates. The assay can be adapted to other volumes, provided the 1:1 ratio of Caspase-Glo® 1 Reagent volume and sample volume is maintained (e.g., 25µl of sample and 25µl Caspase-Glo® 1 Reagent in a 384-well format).

Grow cells in multiwell plates that are adequate for cell culture and compatible with the luminometer being used. Prepare the following reactions to detect caspase-1 activity in cell culture. “Vehicle” refers to the solvent used to dissolve the test compound or protein of interest.

- Blank reaction: vehicle and cell culture medium without cells
- Negative control: vehicle-treated cells in cell culture medium
- Experimental reaction: treated cells in culture medium (to test for inflammasome induction) or inflammasome-induced cells with test compound or protein of interest (to test for inhibition of inflammasome induction)

The blank reaction is used to measure background luminescence associated with the cell culture medium and Caspase-Glo® 1 Reagent. The luminescence values for the blank reaction can be subtracted from values obtained for negative control cells and experimental cells. The negative control reaction is important to determine the background activity of the cell culture system. The experimental reaction monitors caspase-1 activation or inhibition of caspase-1 activation.

Two examples of suitable plate layouts are shown in Figure 5.

Materials to be Supplied by the User

(Solution compositions are provided in Section 8.)

- white, opaque-walled multiwell tissue culture plates (with a clear or solid bottom) compatible with a luminometer
- cells and culture medium
- multichannel pipette or automated pipetting station
- plate shaker for mixing multiwell plates (optional)
- luminometer capable of reading multiwell plates

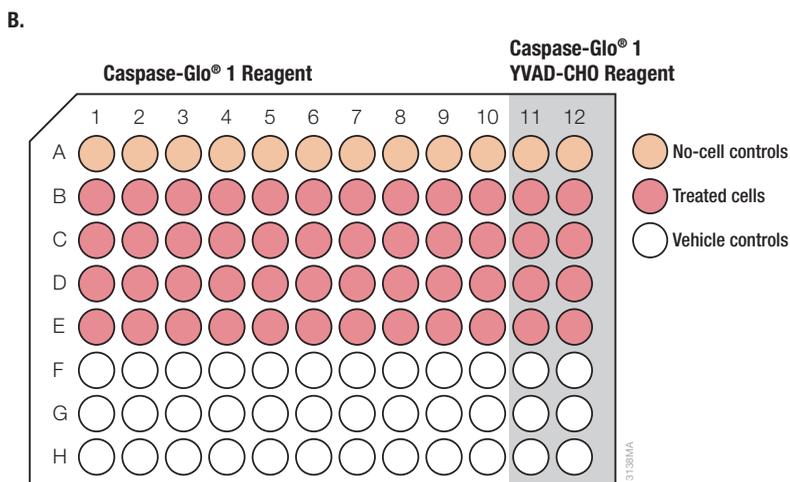
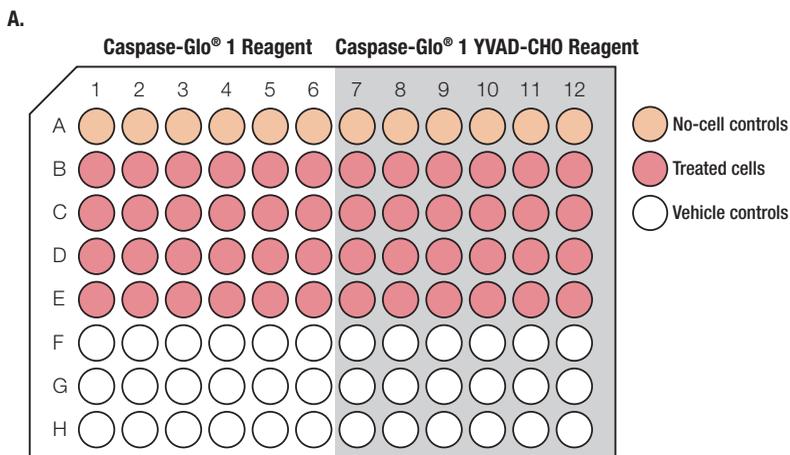


Figure 5. Examples of plate layouts for the Caspase-Glo® 1 Inflammasome Assay. Panel A. The plate layout to test compounds for inflammasome induction. **Panel B.** The plate layout to test inflammasome inhibitors.

4.A. Protocol for Measuring Caspase-1 Activity in Cultured Cells (continued)

1. Before starting the assay, prepare the Caspase-Glo® 1 Reagent and Caspase-Glo® 1 YVAD-CHO Reagent as described in Section 3. Allow reagents to equilibrate to room temperature.
2. Remove 96-well plates containing cells from the incubator, and allow plates to equilibrate at room temperature for 5 minutes.
3. Add 100µl of Caspase-Glo® 1 Reagent to half of the wells of the 96-well plate containing 100µl of blank reaction, negative control cells or treated cells in culture medium.
Note: If you are reusing pipette tips when dispensing reagents, avoid touching pipette tips to wells containing samples to avoid cross-contamination.
4. To the other half of the plate, add 100µl of Caspase-Glo® 1 YVAD-CHO Reagent. Cover the plate with a plate sealer or lid.
5. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds.
Note: Mixing is optional. See Section 6.F.
6. Incubate at room temperature for at least 1 hour to allow the luminescent signal to stabilize.
7. Measure luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.
Note: To confirm that MG-132 inhibition and Ac-YVAD-CHO inhibition are complete, we recommend that you measure luminescence at 60 minutes, 90 minutes and 120 minutes. The plate can be left in the luminometer with kinetic read settings to read every 30 minutes. Once the signal has stabilized, after the MG-132 inhibition and Ac-YVAD-CHO inhibition are complete, the signal half-life is >3 hours (see Figure 12 in Section 6.A.). We recommend that you measure luminescence within 3 hours of adding the Caspase-Glo® Reagent. For batch processing, the signal stability enables reading luminescence anytime between 1–3 hours.

4.B. Protocol for Measuring Released Caspase-1 Activity in Cell Culture Medium

This protocol describes assay setup in a total volume of 100µl using 96-well plates. The assay can be adapted to other volumes provided the 1:1 ratio of Caspase-Glo® 1 Reagent volume to sample volume is used (e.g., 12.5µl of sample and 12.5µl Caspase-Glo® 1 Reagent in a 384-well format).

Example data are shown in Figure 6.

Grow cells in multiwell plates that are adequate for cell culture. If you plan to use the cells for multiplexing with luminescent assays, be sure to use plates that are compatible with the luminometer being used. Prepare the following reactions to detect caspase-1 activity in cell culture. “Vehicle” refers to the solvent used to dissolve the test compound or protein of interest.

- Blank reaction: vehicle and cell culture medium without cells
- Negative control: vehicle-treated cells in cell culture medium
- Experimental reaction: treated cells in culture medium (to test for inflammasome induction) or inflammasome-induced cells with test compound or protein of interest (to test for inhibition of inflammasome induction)

The blank reaction is used to measure background luminescence associated with the cell culture medium and Caspase-Glo® 1 Reagent. The luminescence value for the blank reaction can be subtracted from values obtained for negative control cells and experimental cells. The negative control reaction is important for determining the background activity of the cell culture system. The experimental reaction monitors caspase-1 activation or inhibition of caspase-1 activation.

Materials to be Supplied by the User

(Solution compositions are provided in Section 8.)

- white, opaque-walled multiwell tissue culture plates (with a clear or solid bottom) compatible with a luminometer
- cells and culture medium
- multichannel pipette or automated pipetting station
- plate shaker for mixing multiwell plates (optional)
- luminometer capable of reading multiwell plates

1. Before starting the assay, prepare the Caspase-Glo® 1 Reagent and Caspase-Glo® 1 YVAD-CHO Reagent as described in Section 3. Allow reagents to equilibrate to room temperature.
2. Remove 96-well plates containing cells from the incubator.
3. Transfer 50µl of cell culture medium from each well of the experimental plate to the corresponding well of a new white 96-well plate.
4. Immediately add 50µl Caspase-Glo® 1 Reagent to half of the wells in the 96-well plate containing the transferred culture medium.

Note: If you are reusing pipette tips when dispensing reagent, avoid touching pipette tips to wells containing samples to avoid cross-contamination.

5. To each well in the other half of the plate containing the transferred culture medium, add 50µl of Caspase-Glo® 1 YVAD-CHO Reagent. Avoid touching pipette tips to the wells containing samples to avoid cross-contamination. Cover the plate with a plate sealer or lid.
6. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds.

Note: Mixing is optional. See Section 6.F.

7. Incubate at room temperature for at least 1 hour for the luminescent signal to stabilize.
8. Measure luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.

Note: To confirm that MG-132 inhibition and Ac-YVAD-CHO inhibition are complete, we recommend that you measure luminescence at 60 minutes, 90 minutes and 120 minutes. The plate can be left in the luminometer with kinetic read settings to read every 30 minutes. Once the signal has stabilized, after the MG-132 inhibition and Ac-YVAD-CHO inhibition are complete, the signal half-life is >3 hours (see Figure 12 in Section 6.A.). We recommend that you measure luminescence within 3 hours of adding the Caspase-Glo® 1 Reagent.

4.C. Protocol for Measuring Caspase-1 Activity Using Purified Caspase

This protocol describes assay setup in a total volume of 100µl using 96-well plates. The assay can be easily adapted to different volumes provided the 1:1 ratio of reconstituted Z-WEHD Substrate volume to sample volume is used (e.g., 25µl of sample and 25µl reconstituted Z-WEHD Substrate in a 384-well format).

Prepare the following reactions in multiwell plates to detect caspase-1 activity or inhibition of activity in purified enzyme preparations. “Vehicle” refers to the solvent used to dissolve the test compound.

- Negative control: enzyme dilution buffer and vehicle for test compound or inhibitor, if used
- Positive control: vehicle and purified caspase-1 enzyme in enzyme dilution buffer
- Experimental reaction: test compound and purified caspase-1 enzyme in enzyme dilution buffer

The negative control is used to measure background luminescence associated with the test compound vehicle in the presence of reconstituted Z-WEHD Substrate. The positive control is used to determine the maximum luminescence that can be obtained with the purified caspase-1 enzyme.

Notes:

1. You may need to determine the optimal caspase-1 concentration empirically.
2. Use identical enzyme concentrations for the experimental and positive control reactions.
3. Caspase specific activities and unit definitions can vary widely, depending on the manufacturer.
4. There are two forms of recombinant caspase-1 commercially available. One form is derived from the wild-type caspase-1 sequence, and the other contains a mutation to minimize autoproteolysis. The wild-type caspase-1 is not stable, and the signal appears nonlinear when the enzyme is titrated. The mutated form of caspase-1 gives a linear signal over a very broad range of enzyme concentrations, but the enzyme activity is not very stable, with a half-life of approximately 30 minutes.

Materials to be Supplied by the User

(Solution compositions are provided in Section 8.)

- white, opaque-walled multiwell plates compatible with a luminometer
 - multichannel pipette or automated pipetting station
 - plate shaker for mixing multiwell plates (optional)
 - luminometer capable of reading multiwell plates
 - purified caspase-1 enzyme (e.g., ENZO Cat.# BML-SE168-5000 or #ALX-201-056)
 - a suitable enzyme dilution buffer to dilute purified caspase-1 enzyme, such as the caspase-1 dilution buffer
1. Prepare the reconstituted Z-WEHD Substrate as described in Section 3 (the MG-132 Inhibitor and YVAD-CHO Inhibitor are not required for enzyme activity assays). Allow the reagent to equilibrate to room temperature.
 2. Prepare the negative controls, positive controls and experimental reactions described above in a 96-well plate. Be sure that the final volume in each well is 50µl.
 3. Add 50µl of reconstituted Z-WEHD Substrate to each sample.

Note: If you are reusing pipette tips when dispensing reagent, avoid touching pipette tips to wells containing samples to avoid cross-contamination.

4. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds.
Note: Mixing is optional. See Section 6.F.
5. Incubate at room temperature for 10 minutes.
6. Measure luminescence using a plate-reading luminometer as directed by the luminometer manufacturer.
Note: We recommend that you measure luminescence within 30 minutes of adding the Caspase-Glo® 1 Reagent.

5. Representative Data

The Caspase-Glo® 1 Assay can be used to measure caspase-1 activity directly in cell cultures or released caspase-1 activity in culture medium (Figure 6). Monitoring released caspase-1 activity from culture medium is nondestructive, enabling multiplexing of the biological sample with other assays (Figure 7).

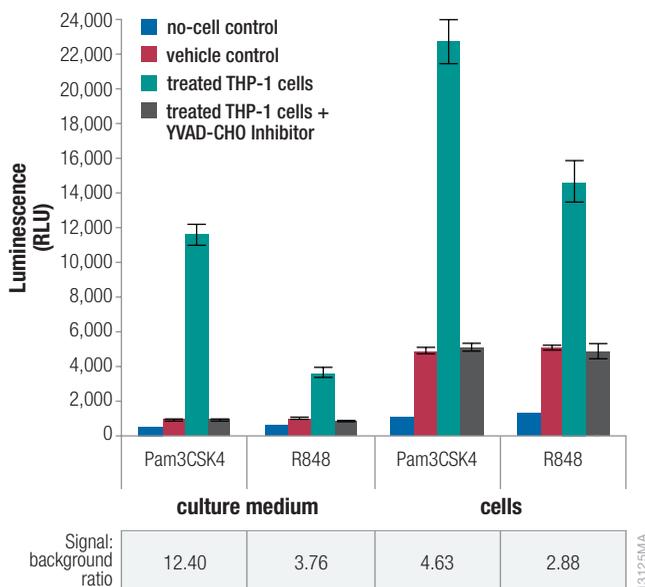


Figure 6. Caspase-Glo® 1 Inflammasome Assay can monitor released caspase-1 in culture medium. THP-1 cells grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂ were differentiated for 2 days with 20nM phorbol-12-myristate-13-acetate (PMA), followed by treatment with either Pam3CSK4 (2µg/ml) or resiquimod (R848, 20µM) for 2 hours. Half of the culture medium (50µl/well) was transferred to a second plate, 50µl/well of Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent was added and luminescence was recorded using a GloMax® Multi+ Detection System as directed in the *GloMax® Multi+ Detection System with Instinct® Software Technical Manual #TM340*. For cells, 100µl/well of reagent was added directly to 100µl/well of cultured cells.

5. Representative Data (continued)

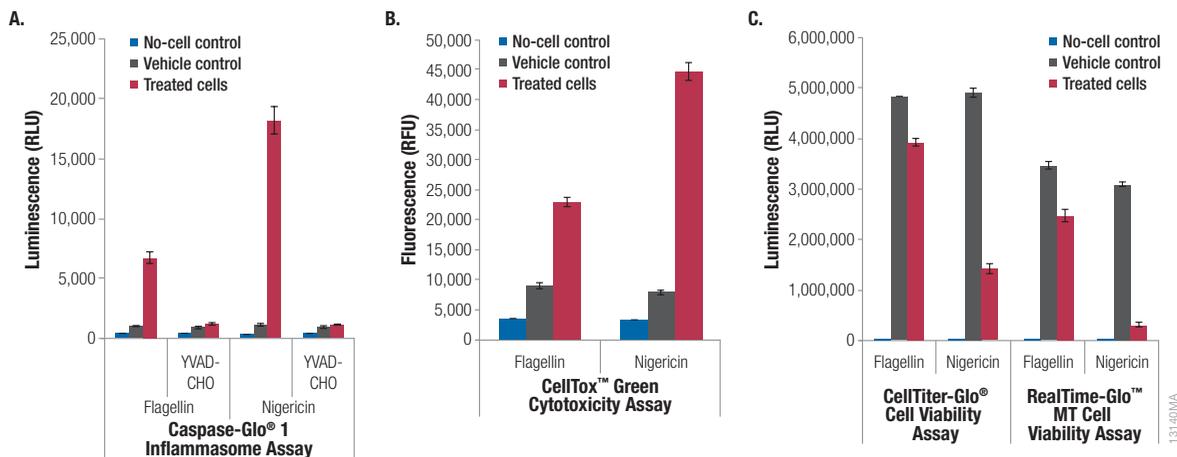


Figure 7. Caspase-Glo[®] 1 Inflammasome Assay multiplexed with cell viability and cell death assays. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂. Cells were added to plates at 5 × 10⁵ cells/ml in 100µl of medium and differentiated with PMA (20nM, 3 days) in 96-well plates followed by treatment with flagellin (1µg/ml, 1 hour) or nigericin (20µM, 2 hours). **Panel A.** Half of the culture medium (50µl) was transferred to a separate plate, and 50µl of Caspase-Glo[®] 1 Reagent or Caspase-Glo[®] 1 YVAD-CHO Reagent was added to each well. Luminescence was recorded after 30 minutes. The original plate with the cells and half of the culture medium was then assayed using the CellTox[™] Green Cytotoxicity Assay, CellTiter-Glo[®] Luminescent Cell Viability Assay or RealTime-Glo[™] MT Cell Viability Assay per the manufacturer's instructions. **Panel B.** CellTox[™] Green Reagent was added to the cells, and fluorescence was recorded after 90 minutes. **Panel C.** Cell viability was monitored with CellTiter-Glo[®] or RealTime-Glo[™] MT Cell Viability Assay. Luminescence was recorded at 10 minutes and 90 minutes, respectively. All luminescence and fluorescence readings were recorded using a GloMax[®] Multi+ Detection System as directed in the *GloMax[®] Multi+ Detection System with Instinct[®] Software Technical Manual #TM340*.

Using this assay system, caspase-1 activation has been demonstrated in THP-1 cells following induction with nigericin, monosodium urate (MSU), α -hemolysin, lipopolysaccharide (LPS), resiquimod (R848), Pam3CSK4 and flagellin in both 96- and 384-well plates (Figures 6–8). Caspase-1 activity also has been measured in bone marrow-derived macrophages from mice (Figure 9), human primary monocytes (Figure 10) and J774A.1 mouse macrophages (Figure 11).

Note: Listed below is a short description of each inflammation inducer used to generate the data in this section.

Staphylococcal α -hemolysin: A bacterial pore-forming toxin that activates inflammasome activity and induces programmed necrosis (pyroptosis; 7, 8).

LPS (lipopolysaccharides): A toll-like receptor 4 (TLR4) agonist that is found in the outer membrane of Gram-negative bacteria and acts as an endotoxin, eliciting a strong immune response (9–11).

R848 (resiquimod): A TLR7/8 agonist that can activate the inflammasome (12).

PAM3CSK4: A synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial LPS. Also a TLR2 agonist, resulting in caspase-1 activation (11).

Flagellin: A TLR5 agonist that also activates the NLRC4/NAIP5 inflammasome intracellularly (13,14).

Nigericin: A microbial toxin that acts as a potassium ionophore; the decrease in intracellular K^+ causes caspase-1 activation (15).

Monosodium urate crystals: The agent that causes acute inflammatory conditions in gout. Crystals cause lysosomal rupture and activation of caspase-1 (16, 17).

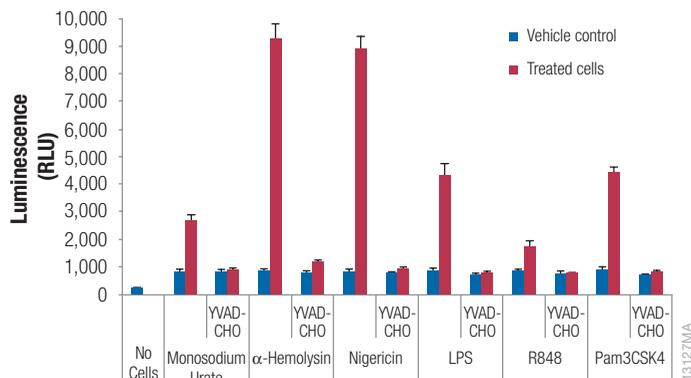


Figure 8. Caspase-Glo® 1 Inflammasome Assay in 384-well plates. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂. Cells were added to 384-well plates at 1.25×10^5 cells/ml in 20 μ l of medium, differentiated with PMA (20nM, 2 days) and then treated with MSU (200 μ g/ml, 4 hours), α -hemolysin (2 μ g/ml, 2.5 hours), nigericin (20 μ M, 2 hours), LPS (Ultrapure, 1 μ g/ml, 2.5 hours), R848 (20 μ M, 2.5 hours), Pam3CSK4 (2 μ g/ml, 1.5 hours) or the appropriate vehicle control. Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent was added (20 μ l/well), and luminescence was measured using a GloMax® Discover System after 90 minutes.

5. Representative Data (continued)

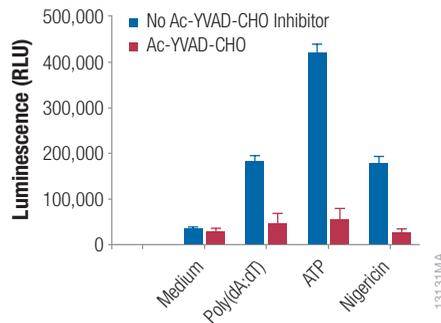


Figure 9. Caspase-Glo® 1 Inflammasome Assay monitors caspase-1 activity in culture medium from bone marrow-derived mouse macrophages. Macrophages were generated by culturing bone marrow cells in DMEM with 20% L929 supernatant for 8 days. Cells were primed with 100ng/ml LPS for 3–8 hours and then stimulated with poly(dA:dT) (double-stranded DNA; 1µg/ml) for 7 hours or with ATP (5mM) or nigericin (10uM) for 1 hour. Cell supernatant was transferred and Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent (50µl/well) was added to the wells, and luminescence was measured on a BioTek Synergy™ microplate reader. (Figure courtesy of Drs. Sivapriya Kailasan Vanaja and Vijay Rathinam, University of Connecticut.)

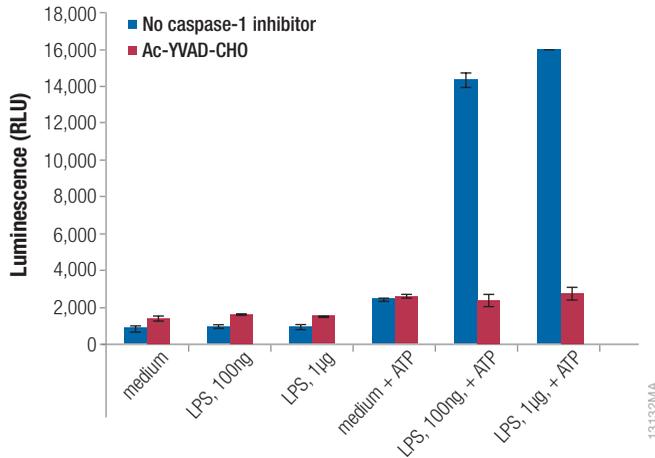


Figure 10. Caspase-Glo® 1 Inflammasome Assay monitors caspase-1 activity in peripheral blood mononuclear cell (PBMC) supernatant. Whole blood was layered on top of Lympholyte Human Cell Separation Media (Cedarlane) in a centrifuge tube and centrifuged for 20 minutes at 800 × g. Using a Pasteur pipette, the buffy coat layer was carefully removed and transferred to a fresh tube containing PBS plus 10mM EDTA and 2% fetal bovine serum (FBS). Cells were washed twice with PBS + EDTA + FBS and then resuspended in RPMI 1640 medium + 10% FBS at 1 × 10⁶ cells/ml. Cells were plated in a 96-well plate and stimulated with or without LPS from *E. coli* 0111:B4 (100ng/ml or 1µg/ml; Sigma-Aldrich) for 3 hours and then subsequently stimulated with ATP (5mM; Bioshop) for 30 minutes. The plate was centrifuged to pellet the cells, and 50µl of each supernatant was transferred to a white 96-well plate. Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent was added to the wells (50µl/well). Luminescence was recorded after 60 minutes. (Figure courtesy of Carlene Petes and Dr. Katrina Gee, Queen’s University, Kingston, Ontario).

6. General Considerations

6.A. Kinetics of Inflammasome Activation and the Caspase-Glo® 1 Inflammasome Assay

The current assays commonly used to monitor activated caspase-1 (e.g., Western blot and ELISA) measure processed caspase-1 (Western blot) and released caspase-1 (ELISA) but do not necessarily monitor active caspase-1. The Caspase-Glo® 1 Inflammasome Assay measures only active caspase-1; therefore, this technology allows you to carefully monitor active caspase-1 after inflammasome activation. Once caspase-1 is activated and released from cells, the activity is quite transient, with a relatively short half-life (18; Figure 11). We have observed this in both THP-1 and J774A.1 cells with a variety of inflammasome inducers. You may need to optimize the assay timing to detect active caspase-1. However, once the lytic Caspase-Glo® 1 Reagent is added to the cultured cells or spent medium, the inflammasome-mediated activated caspase-1 is stabilized (in contrast to recombinant caspase-1), resulting in a continuous, stable signal once the MG-132 inhibition of proteasome is complete (Figure 12). Cell-derived caspase-1 maintains activity, with a half-life >3 hours in the Caspase-Glo® 1 Reagent, providing flexibility when timing luminometer readings.

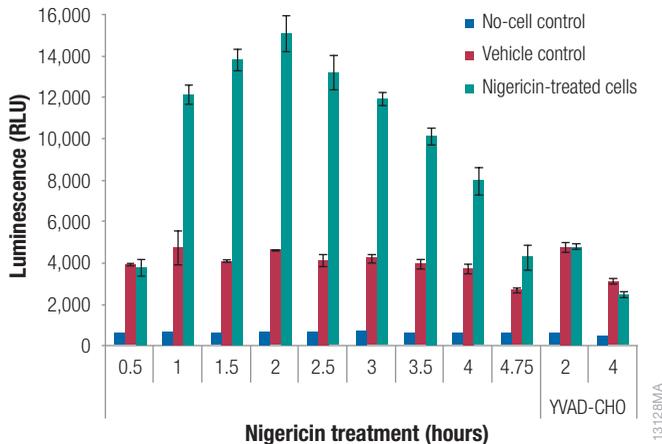


Figure 11. Time course of caspase-1 activation. J774A.1 cells (50,000 cells/well) were grown in DMEM medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂. Cells were added to plates at 4×10^5 cells/ml in 100µl of medium in a 96-well plate and incubated overnight. The next day cells were primed with LPS (500ng/ml) for approximately 4 hours, followed by treatment with nigericin (20µM) or vehicle for the indicated times. Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent was added to the wells (100µl/well). Luminescence was measured using a GloMax® Multi+ Detection System after 2 hours.

6.A. Kinetics of Inflammasome Activation and the Caspase-Glo® 1 Inflammasome Assay (continued)

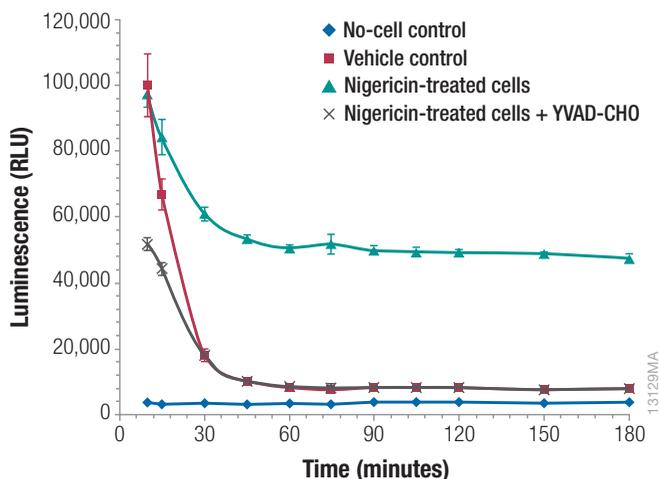
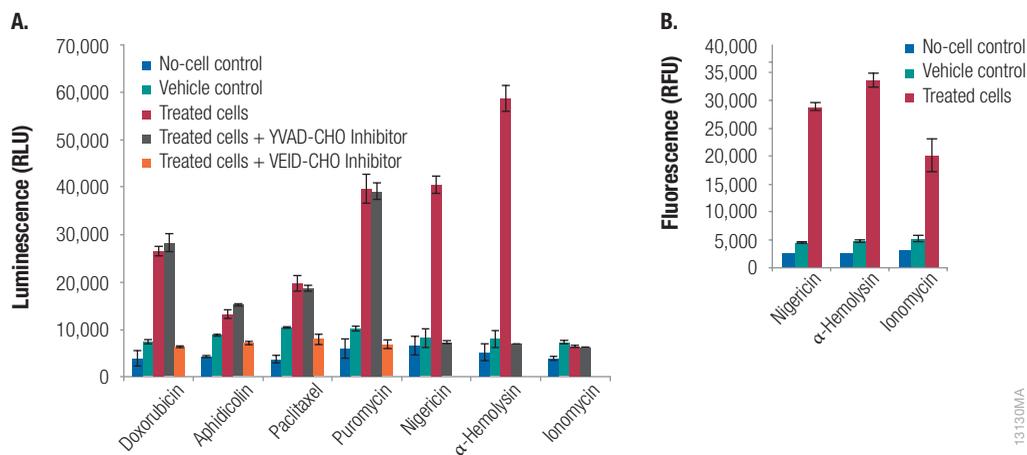


Figure 12. Kinetics of luminescent Caspase-Glo® 1 Assay signal. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂. Cells were added to plates at 5×10^5 cells/ml in 100µl of medium and treated with nigericin (20µM, 2 hours). Caspase-Glo® 1 Reagent or Caspase-Glo® 1 YVAD-CHO Reagent was added to the wells, and luminescence was recorded using a GloMax®-Multi+ Detection System at the indicated times. The red line shows the kinetics of proteasome inhibition, and the gray line illustrates the kinetics of caspase-1 inhibition. The green line illustrates that the Caspase-Glo® 1 signal is stable once proteasome inhibition is complete.

6.B. Caspase-1 Specificity

Caspase tetrapeptide substrates exhibit significant cross-reactivity between caspases (19). In cells, executioner apoptosis caspases are particularly prone to cross-reactivity due to their abundance and strong catalytic activity (20). The Z-WEHD-aminoluciferin substrate has been tested against ten recombinant caspases at equimolar concentrations and was shown to cross-react with caspases 5, 3 and 6 but not caspase 2, 4, 7, 8, 9 or 11 (Figure 4). To detect cross-reactivity, the Caspase-Glo® 1 Assay includes the caspase-1 selective inhibitor, Ac-YVAD-CHO. At a 1µM final concentration, it inhibits 99% of caspase-1 activity but does not substantially inhibit any of the cross-reacting caspases (Figure 4). Caspase-1 specificity is demonstrated by performing two sets of assays in parallel wells: one set with Ac-YVAD-CHO Inhibitor and one without. This requires you to prepare two Caspase-Glo® 1 Reagents: one with Ac-YVAD-CHO at a 2µM concentration (1µM final concentration) and one without, as described in Section 3. Ac-YVAD-CHO inhibition of the luminescent signal confirms that the signal is generated by caspase-1; and conversely, a lack of inhibition indicates that luminescence is not produced by caspase-1 activity (Figures 4 and 13). Caspase-1 inhibition is complete approximately 60 minutes after adding the Caspase-Glo® 1 YVAD-CHO Reagent.



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Figure 13. Caspase-Glo® 1 Inflammasome Assay distinguishes between inflammasome activation, apoptosis and necrosis. Panel A. THP-1 cells grown in RPMI 1640 medium supplemented with 10% FBS in a 37°C incubator with 5% CO₂ were treated with the apoptosis inducers doxorubicin, aphidicolin, paclitaxel and puromycin for 18 hours. THP-1 cells in a second plate were treated with the inflammasome inducers nigericin (20µM) and α -hemolysin (2µg/ml) for 2 hours or with ionomycin (100µM) for 2 hours to induce necrosis. The YVAD-CHO or VEID-CHO inhibitor was added to the Caspase-Glo® 1 Reagent at 2µM for a final concentration of 1µM in the assay. Luminescence was measured using a GloMax® Multi+ Detection System after 60 minutes. Inhibition by YVAD-CHO indicates enzyme activity can be attributed to caspase-1, whereas inhibition by VEID-CHO indicates apoptosis caspase activity that is not caspase-1. **Panel B.** CellTox™ Green Cytotoxicity Reagent was added at the same time as the nigericin, α -hemolysin and ionomycin, and fluorescence was monitored just before adding the Caspase-Glo® 1 Reagent. The increased fluorescent signal with treatment indicates membrane permeability and cell death.

Caspase-Glo® 1 Assay specificity has been tested in cell culture models of inflammasome activation, apoptosis and necrosis. Inflammasome activation in cultured cells produces assay luminescence that is completely inhibited by Ac-YVAD-CHO. Apoptosis in cultured cells generates luminescence that is not inhibited by Ac-YVAD-CHO. Necrosis does not produce a luminescent signal (Figure 13).

Apoptotic cells in your cell culture system will generate a luminescent signal due to caspase-3 and 6 activities. Although the Z-WEHD-aminoluciferin is a relatively poor substrate for apoptotic caspases (19, 21), caspases-3 and 6 are abundant and active enough to be detected with this assay system (20; Figures 4 and 13). Other caspase activities are too low to be detected in cell culture. At the recommended final concentration (1µM), Ac-YVAD-CHO inhibits caspase-1 activity but does not affect caspase-3 or 6 activities (6; Figure 13). Thus, if the signal is not inhibited by 1µM Ac-YVAD-CHO, then the luminescence cannot be attributed to caspase-1.

The concentration of the caspase-1 selective inhibitor, Ac-YVAD-CHO, has been carefully calibrated to discriminate between caspase-1 inflammasome activity and cross-reacting apoptotic caspases in the Caspase-Glo® 1 Reagent. Do not use the inhibitor at higher concentrations or add the inhibitor directly to cells to block inflammasome activation.

6.C. Assay Sensitivity

Luminogenic substrates are typically more sensitive than comparable fluorogenic ones (22–24), and this holds true for caspase-1 substrates (Figure 14). WEHD is the optimal and most sensitive tetrapeptide substrate for caspase-1 (21, 25). To determine assay sensitivity in cells, we titrated THP-1 cells treated with α -hemolysin in 96-well plates and obtained a clear signal with 10,000–80,000 cells/well (Figure 2). We routinely use 50,000 THP-1 cells/well to achieve a robust signal. We also obtained a robust signal with 50,000 J774.1 cells/well (Figure 11). The assay is sensitive enough to clearly detect caspase-1 activity from THP-1 cells treated with a variety of inflammasome inducers in 384-well plates at 12,500 cells/well in 25 μ l of medium (Figure 8). By comparing caspase-3/7 activity monitored with the Caspase-Glo[®] 3/7 Assay and caspase-1 activity measured using the Caspase-Glo[®] 1 Assay, we found that caspase-1 activity is an order of magnitude lower and significantly more transient. Thus, Caspase-Glo[®] 1 luminescence (in relative light units, RLU) was lower than that of other Caspase-Glo[®] Assays, but this difference is due to the transient nature of inflammasome biology and caspase-1 activation and not assay performance.

Cell health will affect assay sensitivity. In cell culture models with high apoptotic activity, the portion of the total signal that can be inhibited by Ac-YVAD-CHO will decrease (see Section 6.B). It is important that cells are as healthy as possible before testing for inflammasome activation. Monitoring released caspase-1 from cell culture medium that is transferred to a new plate, rather than monitoring caspase-1 directly in cultured cells, can improve the signal:background ratio and thus sensitivity (Figure 6). The background signal from untreated controls is typically lower when monitoring released caspase-1 from culture medium.

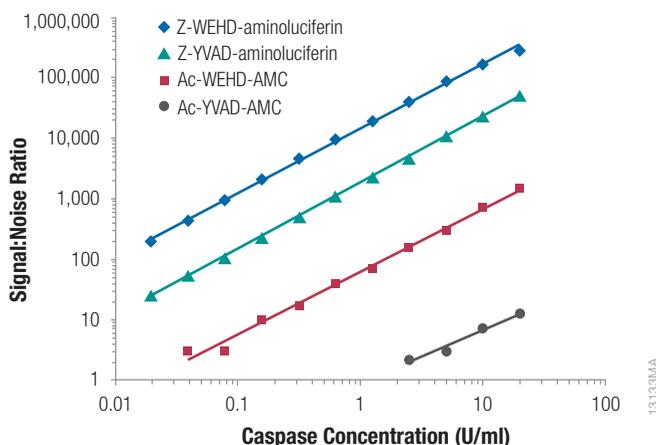


Figure 14. Luminescent and fluorescent assay comparison. Caspase-1 was serially diluted in 10mM HEPES buffer (pH 7.4) with 0.1% Prionex[®] and 2mM DTT to the indicated concentrations in white 96-well plates. For luminescent assays, Luciferin Detection Reagent was reconstituted with Caspase-Glo[®] 1 Buffer, and either Z-WEHD-aminoluciferin or Z-YVAD-aminoluciferin was added to a concentration of 40 μ M. This substrate reagent was added at a 1:1 ratio (v/v), and luminescence was recorded using a GloMax[®]-Multi+ Detection System after 15 minutes. For fluorescent assays, the substrates Ac-WEHD-AMC and Ac-YVAD-AMC were diluted to 40 μ M in 100mM HEPES (pH 7.4), 0.1% Prionex[®] and added at a 1:1 ratio (v/v). Readings were taken over 5 hours using a LabSystems Ascent fluorometer. Data shown represent fluorometer readings at 2.5 hours, which yielded the highest signal:noise ratio. (S:N ratio = Signal – background/standard deviation of background).

6.D. Temperature

The intensity and rate of luminescent signal decay with the Caspase- Glo[®] 1 Assay depend on the rate of decrease in caspase activity and rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction, such as temperature, also affect the intensity of light output and signal stability. For consistent results, be sure to equilibrate assay plates to a constant temperature. In most cases, plates will equilibrate to room temperature during the ≥ 1 -hour incubation prior to luminescence measurement. However, plates removed from a 37°C incubator and placed in tall stacks at room temperature will require longer to equilibrate than plates arranged in a single layer. When batch-mode processing multiple assay plates, include positive and negative controls on each plate to account for any variation due to environmental factors such as temperature.

6.E. Chemicals

The chemical environment of the luciferase reaction will affect enzymatic activity and thus luminescence intensity. We have observed differences in luminescence intensity when using different types of culture media and sera. Solvents used for various chemical compounds may affect the luciferase reaction and thus light output of the assay.

6.F. Mixing

Mixing is not required after adding the Caspase-Glo[®] 1 Reagent. However, if you observe incomplete cell lysis or significant variation in assay signal intensity, we recommend mixing assays to aid cell lysis and improve reproducibility between wells.

6.G. Luminometers

Luminometers from different manufacturers vary in their sensitivity and dynamic range. Consult the operating manual for your luminometer to determine the optimal settings. If necessary, perform preliminary experiments to be sure that the Caspase-Glo[®] 1 Assay signal is within the linear range of your instrument. Individual luminometers may require different gain or sensitivity settings. We recommend that you optimize the gain or sensitivity settings for your instrument.

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8. Composition of Buffers and Solutions

Caspase-1 enzyme dilution buffer

10mM	HEPES buffer (pH 7.4)
0.1%	Prionex®
2mM	DTT



9. Related Products

Viability Assays

Product	Size	Cat.#
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
CellTiter-Glo® 2.0 Assay	10ml	G9241
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Other sizes are available.

Cytotoxicity Assays

Product	Size	Cat.#
CellTox™ Green Cytotoxicity Assay	10ml	G8741
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
LDH-Glo™ Cytotoxicity Assay	10ml	J2380

Other sizes are available.

Mechanism-Based Viability and Cytotoxicity Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml	G6410

Other sizes are available.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011

Other sizes are available.

Metabolism Assays

Product	Size	Cat.#
NAD(P)H-Glo™ Detection System	10ml	G9061
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
Lactate-Glo™ Assay	5ml	J5021
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamine/Glutamate-Glo™ Assay	5ml	J8021

Other sizes are available.

Mitochondrial Toxicity

Product	Size	Cat.#
Mitochondrial ToxGlo™ Assay	10ml	G8000
	100ml	G8001

Oxidative Stress Assays

Product	Size	Cat.#
ROS-Glo™ H ₂ O ₂ Assay	10ml	G8820
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611

Other sizes are available.

Cytochrome P450 Cell-Based Assays

Product	Size	Cat.#
P450-Glo™ CYP1A2 Induction/Inhibition Assay	10ml	V8421
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	10ml	V9001
P450-Glo™ CYP2C9 Assay	10ml	V8791
P450-Glo™ CYP2B6 Assay	10ml	V8321

Other sizes are available.

Detection Instrumentation

Product	Size	Cat.#
GloMax® Discover System	each	GM3000



10. Summary of Changes

The following changes were made in the 5/19 revision of this document:

1. Addition of 100ml kit (Cat.# G9953).
2. Additional note about batch processing and signal stability in Section 4.A.
2. Administrative changes.

^(a)U.S. Pat. Nos. 7,148,030, 7,384,758, 7,666,987 and 8,071,328, Japanese Pat. No. 4451663 and other patents pending.

^(b)European Pat. No. 1131441 and Japanese Pat. No. 4520084.

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