TECHNICAL MANUAL

Caspase-Glo® 3/7 3D Assay

Instructions for Use of Products
G8981, G8982 and G8983

8/20
TM627

Promega

Caspase-Glo® 3/7 3D 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

1. Description

The Caspase-Glo® 3/7 3D Assay[a] is a homogeneous, luminescent assay that measures caspase-3 and -7 activities present in apoptotic, three-dimensional (3D) cell masses. The assay provides a luminogenic caspase-3/7 substrate that contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and 3D cell lysis. Adding the Caspase-Glo® 3/7 3D Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal, produced by luciferase (Figure 1). Luminescence is proportional to the amount of caspase activity present (Figure 2). The Caspase-Glo® 3/7 3D Reagent relies on the inherent properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which has been engineered to tolerate cell lysis components necessary to liberate caspase activity from 3D cell structures.

1. Description (continued)

The Caspase-Glo® 3/7 3D Assay is designed for use with multiwell-plate formats. Cell washing, removing medium and multiple pipetting steps are not required (Figure 3). The caspase and luciferase enzyme activities reach steady state so that the luminescent signal peaks in approximately 30–40 minutes at room temperature and decays slowly and predictably thereafter (Figure 4). The assay also can be multiplexed with other homogeneous assays to measure more than one parameter from a single well (Figure 5).

The Caspase-Glo® 3/7 3D Assay is ideal for characterizing the potency of agents capable of modulating the apoptotic response in 3D structures in 96- and 384-well formats (Figures 6, 7 and 8). The assay can be employed in a wide range of 3D cell models, including spheroids generated by ultra-low attachment surface chemistries and Matrigel® matrix-embedded micromasses.

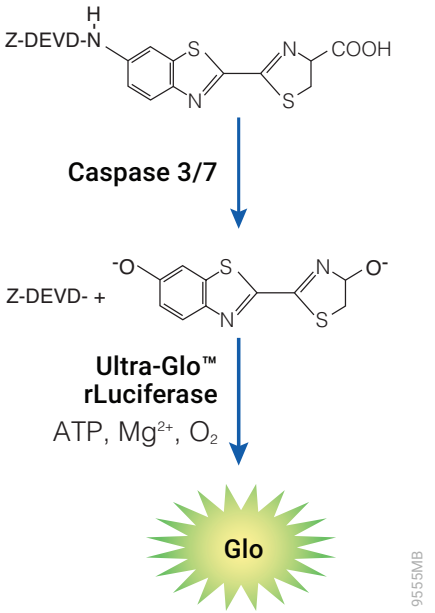

Figure 1. Schematic diagram of the Caspase-Glo® 3/7 3D Assay technology. Following caspase cleavage of the proluciferin DEVD substrate, a substrate for luciferase (aminoluciferin) is released and, in the presence of luciferase and ATP, results in the luciferase reaction and the production of light.

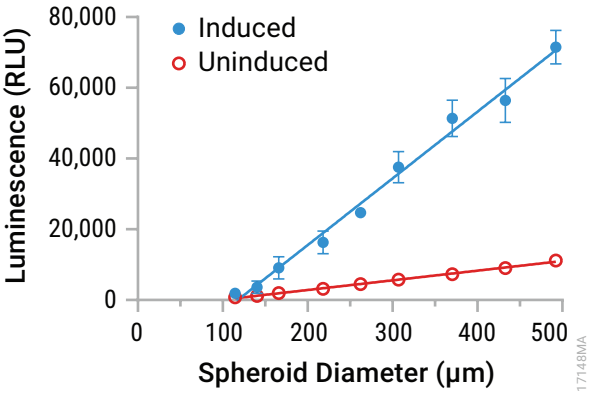

Figure 2. Luminescence is proportional to caspase-3, -7 activity. HCT-116 cells were serially diluted twofold in McCoy's medium supplemented with 10% fetal bovine serum (FBS) and seeded into a 96-well Corning® ultra-low attachment plate. Spheroids formed over 4 days. Average spheroid diameter was determined by brightfield imaging of four replicates per initial seed density. Spent culture medium was removed and replaced with either 10µM bortezomib or vehicle-matched culture medium (induced and uninduced, respectively). Caspase-Glo® 3/7 3D Reagent was added and luminescence measured as described in Section 4.B.

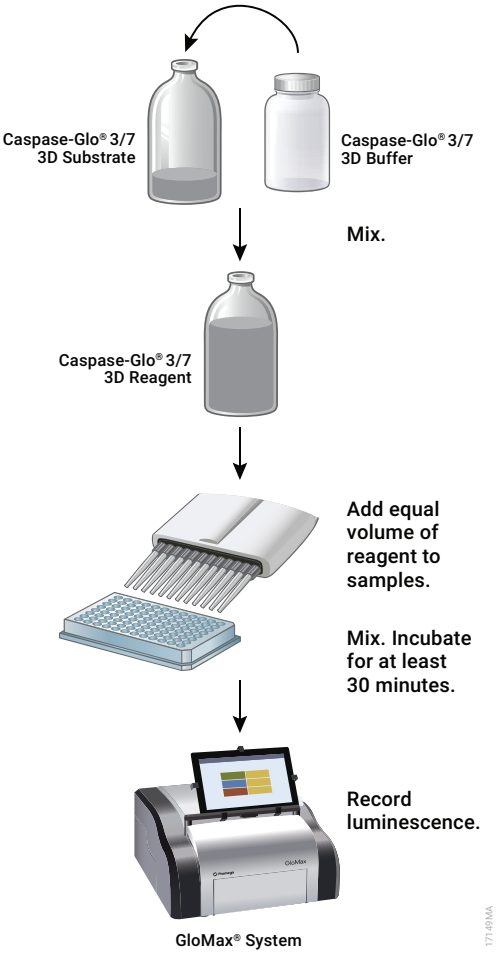

Figure 3. Schematic diagram of the Caspase-Glo® 3/7 3D Assay protocol.

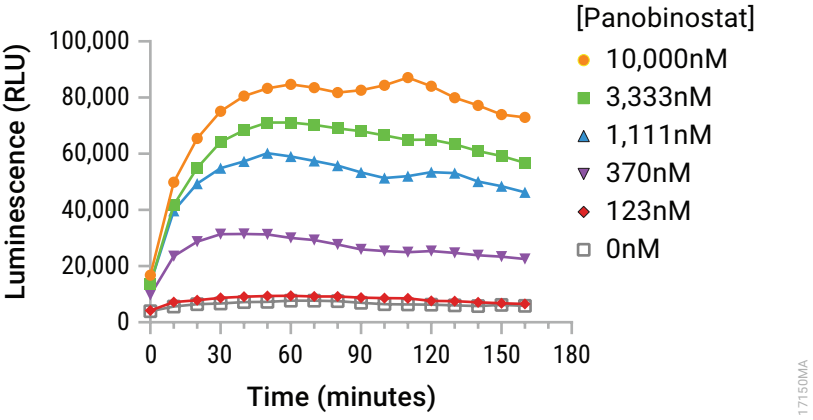

Figure 4. Assay signal kinetics. HCT-116 spheroids (average diameter of 480µm) were treated with serial dilutions of panobinostat or vehicle-matched control for 24 hours. Caspase-Glo® 3/7 3D Reagent was added, the solution mixed briefly and repeated signal collection initiated in a GloMax® Discover Microplate Reader. Signal plateau is typically achieved within 30–40 minutes with a slow and predictable decay thereafter.

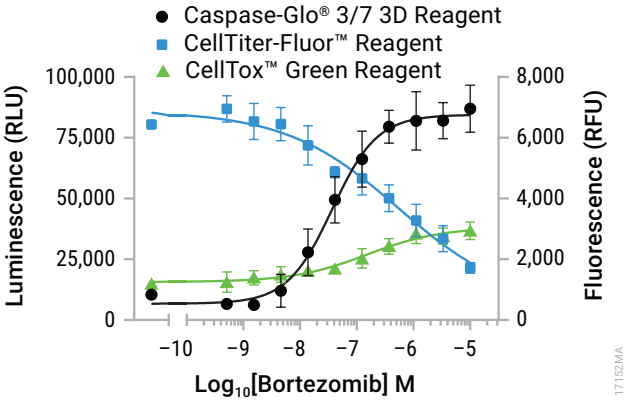

Figure 5. Sequentially multiplexed cell health assay chemistries. A549 spheroids (average diameter of 450µm) were dosed with serial dilutions of bortezomib in the presence of CellTox™ Green Cytotoxicity Reagent for 24 hours. CellTiter-Fluor™ Cell Viability Reagent was delivered to each well and the plate incubated at 37°C for 30 minutes. Fluorescence values resulting from the viability and cytotoxicity measurements were collected at the appropriate wavelengths using a GloMax® Discover Microplate Reader. Caspase-Glo® 3/7 3D Reagent was added, mixed briefly, and luminescence measured after a 30-minute incubation at room temperature. Multiplexed cell health data sets can provide a more complete characterization of an apoptotic response at a given endpoint.

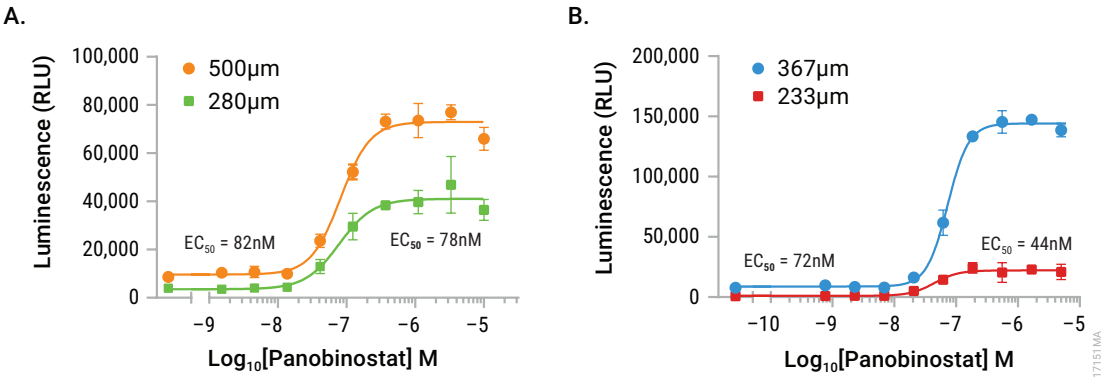

Figure 6. Caspase induction potency in two compact spheroid models. HCT-116 and HepG2 spheroids of differing diameters were created in ultra-low attachment plates over four days. Two sets of spheroids were treated with a master dilution series of panobinostat for 24 hours. Caspase-Glo® 3/7 3D Reagent was added, the solution was mixed briefly and signals collected after 30 minutes of reagent contact. **Panel A.** HCT-116 spheroids produced caspase-activation responses of the same potency that were nearly proportional to spheroid diameter. **Panel B.** HepG2 spheroids produced a differential induction potency signal that was not proportional to spheroid diameter.

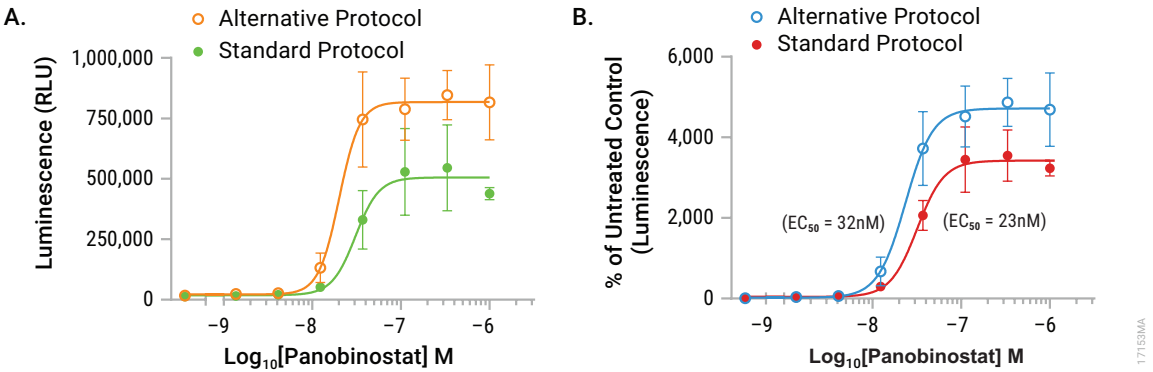

Figure 7. Caspase-induction measurement in a Matrigel® Matrix embedded cell model. HCT-116 cells were embedded in a 4.5mg/ml Matrigel® Solution prepared with McCoy's medium and 10% FBS. Micromasses were allowed to form in the hydrogel for 4 days. Two separate plates were treated with the same serial dilution series of panobinostat for 24 hours. One plate was processed using the standard protocol (Section 4.D) whereas the other plate was processed using the alternative protocol (Section 4.E). **Panel A.** The alternative protocol typically produces about 40% more raw luminescence when compared to the standard protocol. This difference in raw signal can be attributed to removal of phenol red through preprocessing washes, and a reduction in intrinsic hydrogel optical interference. **Panel B.** When luminescence is normalized to protocol-specific uninduced controls, both methods demonstrate dramatic caspase induction signal windows and substantially similar EC_{50} values.

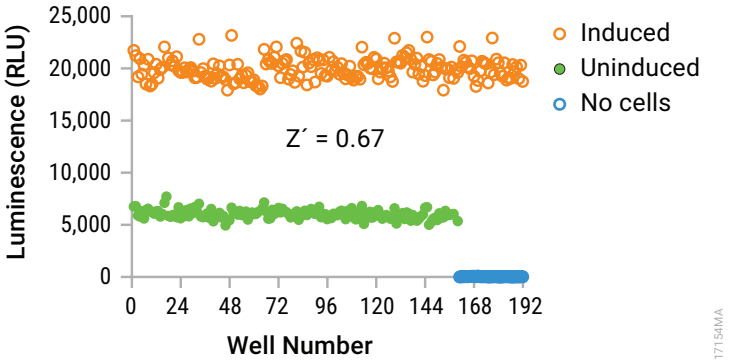

Figure 8. Z´-factor analysis. A549 cells were seeded into a 384-well, plasma-coated, Corning® Elplasia plate and aggregates of cells allowed to form over four days. One-half of the plate was challenged for 24 hours with 10µM bortezomib (induced) whereas the remainder of wells containing cells received vehicle-matched medium control (uninduced). Caspase-Glo ® 3/7 3D Reagent was applied, and luminescence read after 30 minutes at room temperature. The signal resulting from the reagent only (no cells) was plotted with induced and uninduced controls. A Z´-factor calculation of 0.67 (1) suggests high-throughput suitability.

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2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Caspase-Glo® 3/7 3D Assay	**10ml**	**G8981**

Each 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® 3/7 3D Buffer
- 1 bottle Caspase-Glo® 3/7 3D Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
Caspase-Glo® 3/7 3D Assay	**10 × 10ml**	**G8983**

Each 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:

- 10 × 10ml Caspase-Glo® 3/7 3D Buffer
- 10 bottles Caspase-Glo® 3/7 3D Substrate (lyophilized)

PRODUCT	SIZE	CAT.#
Caspase-Glo® 3/7 3D Assay	**100ml**	**G8982**

Each 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® 3/7 3D Buffer
- 1 bottle Caspase-Glo® 3/7 3D Substrate (lyophilized)

Storage Conditions: Store the Caspase-Glo® 3/7 3D Substrate and Caspase-Glo® 3/7 3D Buffer at –30°C to –10°C protected from light. The Caspase-Glo® 3/7 3D Buffer may be thawed and stored at +2°C to +10°C for 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

1. Equilibrate the Caspase-Glo® 3/7 3D Buffer and lyophilized Caspase-Glo® 3/7 3D Substrate to room temperature before use.
2. Transfer the contents of the Caspase-Glo® 3/7 3D Buffer bottle into the amber bottle containing Caspase-Glo® 3/7 3D Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 3D Reagent. Buffer volumes are 10ml for Cat.# G8981 and Cat.# G8983, and 100ml for Cat.# G8982.

Storage: Store the reconstituted Caspase-Glo® 3/7 3D Reagent at +2°C to +10°C for up to 3 days with no loss of activity compared to that of freshly prepared reagent. Reconstituted reagent stored at +2°C to +10°C for 1 week will give a signal approximately 90% of the signal obtained with freshly prepared reagent, while reconstituted reagent stored at +2°C to +10°C for 4 weeks will give a signal approximately 75% of that obtained with freshly prepared reagent. Reconstituted reagent that has been refrozen and stored at −30°C to −10°C for 1 week will give a signal approximately 75% of that of newly prepared reagent, and refrozen reagent stored at −30°C to −10°C for 4 weeks will give a signal approximately 60% of that of newly prepared reagent.

4. Detection of Caspase-3 and -7 Activities in 3D Cell Models

Directions are given for performing the assay in a total volume of 200µl using 96-well plates. However, the assay can be adapted to other volumes, provided the 1:1 ratio of Caspase-Glo® 3/7 3D Reagent volume to sample volume is used (e.g., 25µl of sample and 25µl Caspase-Glo® 3/7 3D Reagent in a 384-well format).

Materials to Be Supplied by the User

- multiwell plates used specifically for 3D cell culture and compatible with the luminometer being used; For example:
  - Corning® microplates (Cat.# 3917) or equivalent for Matrigel® embedded micromasses
  - Corning® ultra-low attachment (ULA) microplates (Cat.# 4442) or equivalent for spheroid generation
  - Corning® Elplasia (Cat.# 4447) or equivalent for 384-well density
- luminometer capable of reading multiwell plates (e.g., GloMax™ Discover, Cat.# GM3000)
- **optional:** CellTox™ Green Cytotoxicity Assay (Cat.# G8741) and/or CellTiter-Fluor™ Cell Viability Assay (Cat.# G6081) for conducting sequential cell health multiplexing (Section 4.C)
- **optional:** Corning® Cell Recovery Solution (Cat.# 354253) for conducting alternative Matrigel® protocol (Section 4.E)

4.A. Assay Conditions

Institute the following treatments and controls to detect caspase-3 and -7 activities in 3D cell cultures. Grow cells in multiwell plates that are designed for your specific 3D application and compatible with the luminometer being used. "Vehicle" refers to the solvent used to dissolve the drug or protein of interest.

- Cell-free control: Caspase-Glo® 3/7 3D Reagent, vehicle and cell culture medium without cells
- Uninduced negative control: Caspase-Glo® 3/7 3D Reagent and vehicle-treated cells in medium
- Induced positive control: Caspase-Glo® 3/7 3D Reagent and reference inducer-treated cells in medium
- Test assays: Caspase-Glo® 3/7 3D Reagent and treated cells in medium

The cell-free control is used to measure background luminescence associated with the cell culture components and Caspase-Glo® 3/7 3D Reagent. Subtract the value for the cell-free control from all other values. Uninduced negative controls and induced positive controls are important for determining the basal caspase activity of the cell culture system and the potential for apoptotic inducibility of the cell model, respectively.

Due to the sensitive nature of the Caspase-Glo® 3/7 3D Assay, avoid contamination with solutions that may contain luciferin.

Notes:

1. Empirically determine the optimal seeding density required to create spheroids or microtissues of the desired diameter. We recommend initial seeding densities that can be supported in a 96-well plate culture well during the three- to four-day period required to generate the 3D masses. Depending on cell line-specific doubling times, this is typically <2,000 cells per well.
2. Apoptosis induction kinetics and response magnitude will be treatment- and culture system-dependent. Time-course experiments may be required to optimize peak caspase activation.
3. Use identical cell number and volume for the assay and the negative control samples.
4. The optimal Caspase-Glo® 3/7 3D Reagent contact period for best signal will depend upon the culture system, but typically will be reached within 30–40 minutes. We do not recommend reagent incubation times greater than 3 hours. In general, the luminescent signal will decay about 5–10% per hour after signal plateau (Figure 4).

4.B. Standard Protocol for Spheroids in 96-well Corning® Ultra-Low Attachment Plates

1. Before starting the assay, prepare the Caspase-Glo® 3/7 3D Reagent (see Section 3). Equilibrate the reagent to room temperature. Mix well.
2. Remove the 96-well plates containing treated cells from the incubator and equilibrate plates to room temperature.
3. Add 100µl of Caspase-Glo® 3/7 3D Reagent to each well of the 96-well plate containing 100µl of cell-free, uninduced negative and induced positive control cells, or test treated cells in culture medium.

   Note: If you are reusing pipette tips, do not touch the tips to the wells containing samples to avoid cross-contamination
4. Mix the well contents using a plate shaker at 500rpm for a full 30 seconds. Incubate at room temperature for at least 30 minutes. The plates can be incubated up to 3 hours for convenience, but there will be a gradual decrease in assay performance.

   Note: Temperature fluctuations will affect the luminescence reading. If the room temperature fluctuates, use a constant-temperature incubator. For details see Section 6.
5. Measure luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer.

4.C. Multiplexed Cell Health Assay Protocol for Spheroids

Apoptosis induction responses are dependent on the mode-of-action and potency of the stimulus and the cell model contact period. Therefore, it is often desirable to incorporate other assay chemistries to assess cell health when conducting endpoint, caspase-activation assays. In Figure 6, CellTox™ Green Cytotoxicity Assay and CellTiter-Fluor™ Viability Assay reagents are added and the resulting spectrally-distinct signals measured using a fluorometer prior to adding the Caspase-Glo® 3/7 3D Reagent. Together, the fluorescent and luminescent assay responses help to determine the relationship between anti-proliferative effects, caspase activation and loss of membrane integrity at the endpoint exposure period chosen.

Note: This protocol outlines the sequential steps of multiplexing the CellTox™ Green Cytotoxicity Assay and CellTiter-Fluor™ Viability Assay chemistries. Refer to the *CellTox™ Green Cytotoxicity Assay Technical Manual,* #TM375 and *CellTiter-Fluor™ Cell Viability Assay Technical Bulletin*, #TB371, respectively, for more complete guidance, tips and protocols.

4.C. Multiplexed Cell Health Assay Protocol for Spheroids (continued)

1. Thaw CellTox™ Green Cytotoxicity Assay Dye (1,000X) by immersing in a 37°C water bath.
2. Prepare serial dilutions of test article in complete cell culture medium containing 2X CellTox™ Green Cytotoxicity Dye.
3. Add an equal volume of the dilution series containing CellTox™ Green Cytotoxicity Dye to each well of a plate containing spheroids. For 96-well applications, 50µl of test article dilution series should be added to each well.
4. Incubate spheroids for the desired exposure period.
5. Thaw CellTiter-Fluor™ Cell Viability Assay components by immersing in a 37°C water bath.
6. Create a 5X CellTiter-Fluor™ Cell Viability Assay solution by adding 10µl of the GF-AFC substrate to 2,000µl of Assay Buffer.
7. Dispense 20µl of 5X reagent (Step 6) to all wells of the treated spheroid plate. Mix briefly at 500rpm and incubate at 37°C for at least 30 minutes.
8. Collect fluorescence data at Ex_{400}/Em_{505} for cell viability (CellTiter-Fluor™ Assay) and Ex_{485}/Em_{520} for cytotoxicity (CellTox™ Green Assay).
9. Proceed to the protocol described in Section 4.B to collect caspase activation data.

4.D. Standard Protocol for 3D Microtissues Embedded in Matrigel® Matrix

Matrigel® matrix is a complex and variable composition of basement membrane matrices that form semi-solid hydrogels at 37°C. Matrigel therefore complicates Caspase-Glo® 3/7 3D data quality by adversely affecting reagent penetration and by introducing optical interference. You can expect about 40–60% loss in luminescence when compared to similarly treated cells in the absence of Matrigel. If this luminescent signal loss is not tolerable, we suggest implementing the preprocessing step in the alternative protocol in Section 4.E.

1. Before starting the assay, prepare the Caspase-Glo® 3/7 3D Reagent (see Section 3). Equilibrate the reagent to room temperature. Mix well.
2. Remove plates containing 3D Matrigel® embedded and treated cells from the incubator and equilibrate plates to room temperature.
3. Add a volume of Caspase-Glo® 3/7 3D Reagent equal to the volume of medium in the well.

   Note: If you are reusing pipette tips, do not touch the tips to the wells containing samples to avoid cross-contamination.
4. Mix the well contents using a plate shaker at 500rpm for a full 30 seconds. Incubate at room temperature for at least 30 minutes. The plates can be incubated for up to 3 hours for convenience, but there will be a gradual decrease in assay performance with longer incubation times.

   Note: Temperature fluctuations will affect the luminescence reading. If the room temperature fluctuates, use a constant-temperature incubator. See Section 6.
5. Measure the luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer.

4.E. Alternative Protocol for 3D Microtissues Embedded in Matrigel® Matrix

This protocol incorporates a preprocessing step with Corning® Cell Recovery Solution that reduces structural organization of the formed hydrogel and promotes depolymerization for more optimal caspase extraction and measurement. Additional sample manipulation and hands-on time are required.

1. When the test article exposure is complete, remove the fluid phase from the embedded cell model by carefully aspirating with a multichannel pipet or robotic liquid handler.
2. Add an equal volume of chilled (2°C to 8°C) Corning® Cell Recovery Solution to each well. For most 96-well applications, this volume will be 50µl. Cover the plate with a lid or sealer.
3. Place the plate on an ice bath for 1 hour to depolymerize the hydrogel.
4. During the Corning® Cell Recovery Solution incubation in Step 3, prepare the Caspase-Glo® 3/7 3D Reagent (see Section 3). Mix well, then place the reagent on ice.
5. Add an equal volume of ice-cold Caspase-Glo® 3/7 3D Reagent to each well of the plate.

   Note: If you are reusing pipette tips, do not touch the tips to the wells containing samples to avoid cross-contamination.
6. Mix the well contents using a plate shaker at 500rpm for a full 30 seconds. Incubate at room temperature for at least 30 minutes. The plates can be incubated for up to 3 hours for convenience, but there will be a gradual decrease in assay performance with longer incubation times.

   Note: Temperature fluctuations will affect the luminescence reading. If the room temperature fluctuates, use a constant-temperature incubator. See Section 6.
7. Measure the luminescence of each sample in a plate-reading luminometer as directed by the luminometer manufacturer.

5. General Considerations

Sensitivity

Maximum assay sensitivity is achieved when the caspase and luciferase activities reach steady state. Under typical conditions, steady state is reached within 30–40 minutes. The time required for the assay to reach steady state may vary, depending upon the cell culture system used and ambient temperature. The Caspase-Glo® 3/7 3D Reagent can be incubated with samples for up to 3 hours, but often does not improve the signal-to-background ratio achieved in the first 30 minutes.

5. General Considerations (continued)

Temperature

The intensity and rate of decay of the luminescent signal from the Caspase-Glo® 3/7 3D Assay depends on the rate of decrease in caspase activity and the rate of the luciferase reaction. Environmental factors that affect the rate of the luciferase reaction also will affect the intensity of light output and the stability of the luminescent signal. Temperature can affect the rate of this enzymatic assay and thus the light output. For consistent results, equilibrate assay plates to a constant temperature before performing the assay. For batch-mode processing of multiple assay plates, positive and negative controls should be included for each plate. Additionally, 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 time for equilibration than plates arranged in a single layer.

Assay Interferences

The chemical environment of the luciferase reaction can affect enzymatic activity and thus luminescence intensity. Differences in luminescence intensity have been observed using different types of culture media and sera. Solvents used for solubilization of chemical compounds may affect the luciferase reaction and thus the light output from the assay. Dimethyl-sulfoxide (DMSO) has been tested at final concentrations of up to 10% in the assay and found to have a minimal effect on light output.

Optical interferences also influence luminescent intensity. Although typically non-limiting, phenol red present in culture media can reduce raw luminescence values. 3D scaffolding matrices like Matrigel® can change the refractive index and reduce luminescence.

Mixing

Mixing is essential after adding the Caspase-Glo® 3/7 3D Reagent to aid in cell lysis and release of caspases 3 and 7. The caspase activity present in most microtissues or spheroids can be extracted by the reagent after a 30-second mix at 500rpm. Longer mix times do not appreciably improve caspase extraction and can be detrimental to overall luminescence.

Luminometers

For highly sensitive luminometric assays, the luminometer model and settings greatly affect the quality of the data obtained. Luminometers from different manufacturers vary in their sensitivity and dynamic range. If you are not using a GloMax® Luminometer, consult the operating manual for your luminometer to determine the optimal settings. The limits should be verified on each instrument before analysis of experimental samples. The assay should be linear in some portion of the detection range of the instrument used. Within an individual luminometer there may be different gain/sensitivity settings. We recommend that you optimize the gain/sensitivity settings.

We recommend the GloMax® family of luminometers. These instruments do not require gain adjustments to achieve optimal sensitivity and dynamic range. The GloMax® family of luminometers are preloaded with Promega product protocols for ease of use.

6. Reference

1. Zhang, Ji-Hu *et. al.* (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening* **4**, 67–73.

7. Related Products

Apoptosis Products

Product	Size	Cat.#
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml	G6410
Caspase-Glo® 8 Assay	100ml	G8202
Caspase-Glo® 9 Assay	100ml	G8212
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml	G7791
CaspACE™ Assay System, Colorimetric	100 assays	G7220
Caspase Inhibitor Z-VAD-FMK	50µl	G7231
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
DeadEnd™ Colorimetric TUNEL System	20 reactions	G7360
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
rhTNF-α	10µg	G5241
Terminal Deoxynucleotidyl Transferase, Recombinant	300u	M1871

Cell Viability Assays

Product	Size	Cat.#
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Glo® 2.0 Assay	10ml	G9241
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

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7. Related Products (continued)

Cytotoxicity Assays

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (measures release of LDH)	1,000–4,000 assays	G7891
CytoTox 96® Non-Radioactive Cytotoxicity Assay (measures release of LDH)	1,000 assays	G1780
CellTox™ Green Cytotoxicity Assay	10ml	G8741

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611
ROS-Glo™ H_2O_2 Assay	10ml	G8820
Mitochondrial ToxGlo™ Assay	10ml	G8000

Energy Metabolism Assays

Product	Size	Cat.#
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
Glucose Uptake-Glo™ Assay	5ml	J1341

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
GloMax® Navigator System	1 each	GM2000

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

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