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Technical Manual

ApoLive-Glo™ Multiplex Assay

INSTRUCTIONS FOR USE OF PRODUCTS G6410 AND G6411.

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ApoLive-Glo™ Multiplex Assay

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1. Introduction

The ApoLive-Glo™ Multiplex Assay[a–d] combines two assay chemistries to assess viability and caspase activation events within a single assay well. The first part of the assay measures the activity of a protease marker of cell viability. The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-amino fluorocoumarin; GF-AFC). The substrate enters intact cells, where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells (Figure 1). This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium (1).

The second part of the assay uses the Caspase-Glo® Assay technology to detect caspase-3/7 activation, which is a key biomarker of apoptosis. The Caspase-Glo® 3/7 Assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding the Caspase-Glo® 3/7 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 2; 2). Luminescence is proportional to the amount of caspase activity present. The Caspase-Glo® 3/7 Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to generate a stable "glow-type" luminescent signal and improve performance across a wide range of assay conditions.

Advantages of the ApoLive-Glo™ Multiplex Assay:

- **Measure Viability and Apoptosis in the Same Sample Well:** Accurately determine the mechanism of cell death in less time with less sample.
- **Easy to Implement:** The assay uses a simple sequential "add-mix-read" format (Figure 3).
- **Normalize Caspase Data with Viability Control:** The ratio of caspase activity to viable cells is useful for determining the extent of caspase activation and for normalizing cell numbers.
- **Flexible and Easily Automated:** The volumes of each assay component can be scaled to meet throughtput needs, and the assay is amenable to automation in 96- and 384-well plates.
- **Reveal cell death even if the window of caspase activity is missed.**

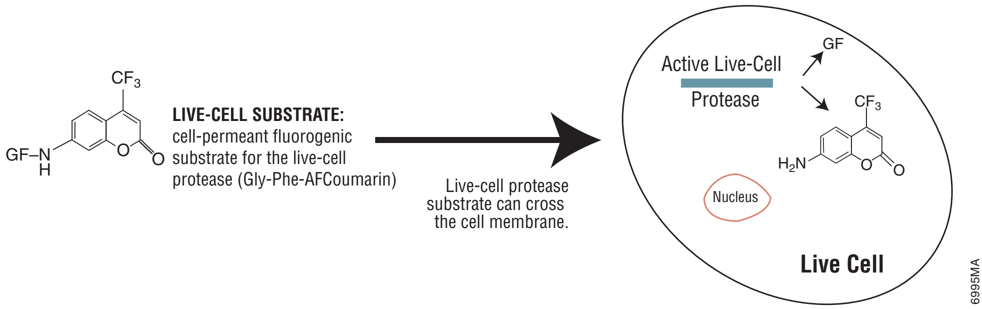

Figure 1. Cell viability assay chemistry. The cell-permeant substrate enters the cell, where it is cleaved by the live-cell protease activity to produce the fluorescent AFC. The live-cell protease is labile in membrane-compromised cells and cannot cleave the substrate.

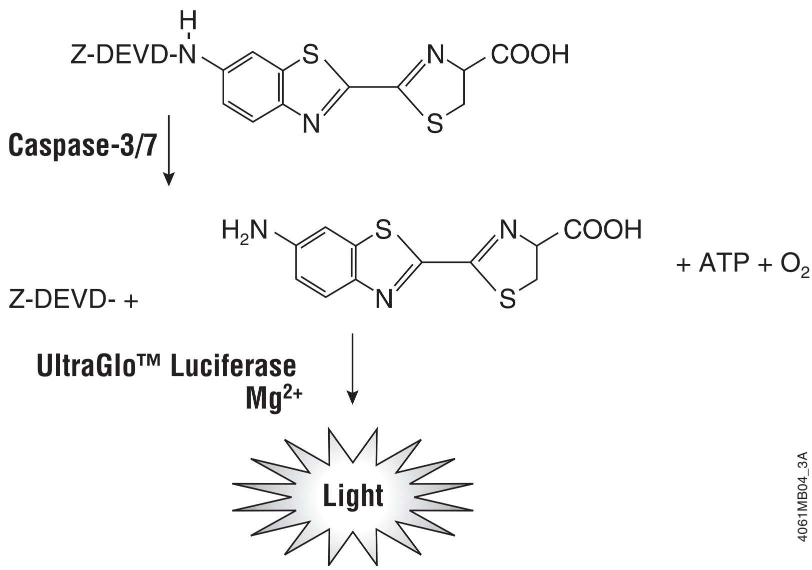

Figure 2. Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence. Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

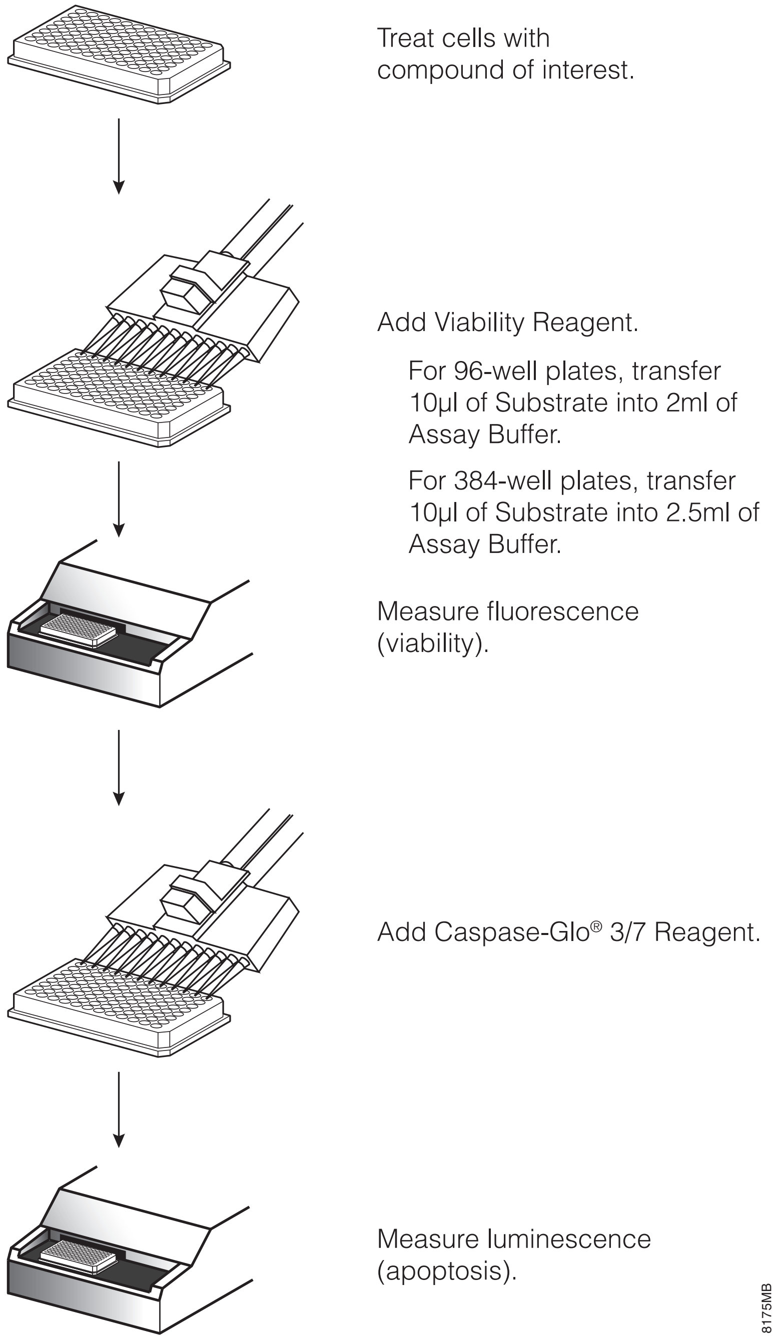

Figure 3. Schematic diagram of the ApoLive-Glo™ Multiplex Assay. Cell viability fluorescence is measured at $400_{Ex}/505_{Em}$, while caspase activity is determined by the luminescence measurement.

2. Product Components and Storage Conditions

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

For in vitro use only. Cat.# G6410 contains sufficient reagents for 100 assays in a 96-well plate format or 400 assays in a 384-well format. Includes:

- 1 × 10ml Assay Buffer
- 1 × 10µl GF-AFC Substrate (100mM in DMSO)
- 1 × 10ml Caspase-Glo® 3/7 Buffer
- 1 bottle Caspase-Glo® 3/7 Substrate (lyophilized)

Product	Size	Cat.#
ApoLive-Glo™ Multiplex Assay	5 × 10ml	G6411

For in vitro use only. Cat.# G6411 contains sufficient reagents for 500 assays in a 96-well plate format or 2,000 assays in a 384-well format. Includes:

- 2 × 10ml Assay Buffer
- 1 × 50µl GF-AFC Substrate (100mM in DMSO)
- 5 × 10ml Caspase-Glo® 3/7 Buffer
- 5 bottles Caspase-Glo® 3/7 Substrate (lyophilized)

Storage Conditions: Store all components at –20°C protected from light. See product label for expiration date.

3. Before You Begin

3.A. Notes

1. Before starting the assay, prepare the Viability Reagent and Caspase-Glo® 3/7 Reagent as directed.
2. Because of the sensitivity of this assay, be careful not to touch pipette tips to the wells containing samples to avoid cross-contamination, particularly if you are reusing pipette tips.
3. Between dispensings, cover the plate with a lid or plate seal to minimize exposure to contaminants in the air.
4. Temperature fluctuations can affect the luminescence readings. If the room temperature fluctuates, use a constant-temperature incubator.
5. Total incubation time for the Caspase-Glo® 3/7 Assay depends upon the culture system, but typically peak luminescent signal will be reached in 1–2 hours. For optimal results, the maximum recommended incubation time is 3 hours. In general, the luminescent signal remaining at 3 hours is greater than 70% of peak luminescence.

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3.B. Reagent Preparation and Storage of Prepared Reagents

1. Thaw each assay component as follows:
   - Assay Buffer: 37°C water bath
   - GF-AFC Substrate: 37°C water bath
   - Caspase-Glo® 3/7 Buffer: Room temperature
   - Caspase-Glo® 3/7 Substrate: Room temperature
2. Transfer the contents of the GF-AFC Substrate into Assay Buffer. For 96-well plates, transfer 10µl of substrate into 2ml of Assay Buffer. For standard 384-well plates, transfer 10µl of substrate into 2.5ml of Assay Buffer. Mix the Assay Buffer containing substrates by vortexing the contents until the substrate is thoroughly dissolved. This mixture will be referred to as the Viability Reagent.

   Note: Once prepared, the Viability Reagent should be used **within 24 hours** if stored at room temperature. Unused Viability Reagent can be stored at 4°C for up to 7 days with no appreciable loss of activity.

3. Transfer the contents of one Caspase-Glo® 3/7 Buffer bottle into one amber bottle containing Caspase-Glo® 3/7 Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 Reagent (~20 seconds).

   Note: Reconstituted Caspase-Glo® 3/7 Reagent can be stored according to the table below.

Storage Temperature	Signal Intensity Compared to Freshly Prepared Reagent
4°C	Up to 3 days with no signal loss
	Stored for 1 week = ~90% signal
	Stored for 4 weeks = ~75% signal
–20°C	Stored up to 1 week = ~75% signal
	Stored up to 4 weeks = ~60% signal

3.C. Recommended Controls

No-Cell Control: Set up triplicate wells with medium but without cells to serve as the negative control for determining background fluorescence and luminescence.

Untreated Cells Control: Set up triplicate wells with untreated cells to serve as a vehicle control. Add the same percent solvent and medium vehicle used to deliver the test compounds to the vehicle control wells.

Optional Test Compound Control: Set up triplicate wells without cells containing the vehicle and test compound to test for possible interference with the assay chemistries (3,4).

Positive Controls:

- **Reduced Cell Viability:** Set up triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system (e.g., final concentration of 30µg/ml digitonin for 15 minutes).
- **Necrosis:** Set up triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system (e.g., final concentration of 100µM ionomycin for 4–6 hours).
- **Apoptosis:** Set up triplicate wells containing cells treated with a compound known to induce apoptosis in the cells used in your model system (e.g., final concentration of 10µM staurosporine for 6 hours).

Note: Be sure to use identical cell numbers and volumes for the assay and the control samples. **You may need to determine empirically the optimal cell number, apoptosis induction treatment and incubation time for your cell culture system.** We recommend using <20,000 cells per well in a 96-well plate and <5,000 cells per well in a 384-well plate.

3.D. Recommended Control Experiment (96-well format)

1. Choose the control compounds (ionomycin or staurosporine or both) appropriate for your experiment. Use 200µM ionomycin and 20µM staurosporine as the starting concentration.

2. Prepare a serial dilution of the control compound (see Figure 4 for plate layout).

   a. Add 50µl of cell culture medium (e.g., RPMI 1640 + 10% FBS) to columns 2–12 of a 96-well assay plate.

   b. Add 50µl of control compound to wells in both columns 1 and 2. Column 1 now contains 50µl, while column 2 contains 100µl. Mix the contents of column 2 by pipetting.

   c. Transfer 50µl from column 2 wells into column 3 wells and mix. Repeat this transfer of 50µl, mixing after each transfer, until column 10. Discard the extra 50µl removed from column 10. All columns should contain 50µl. This creates twofold serial dilutions from columns 1–10.

3.D. Recommended Control Experiment (96-well format; continued)

3. Prepare Jurkat cells at a concentration of 200,000 cells/ml, and dispense 50µl (a total of 10,000 cells/well) to all wells except column 12. This step creates the "Untreated Cells Control" wells in column 11.
4. Add 50µl of medium and vehicle to column 12 to create the "No-cell control" wells (background control). The final volume in all wells will be 100µl.
5. Incubate the cells for 6 hours at 37°C.
6. Add 20µl of Viability Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).
7. Incubate for at least 30 minutes at 37°C.
8. Measure fluorescence at the following wavelengths: $400_{Ex}/505_{Em}$ (viability).
9. Add 100µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).
10. Incubate for at least 30 minutes at room temperature.
11. Measure luminescence (apoptosis).

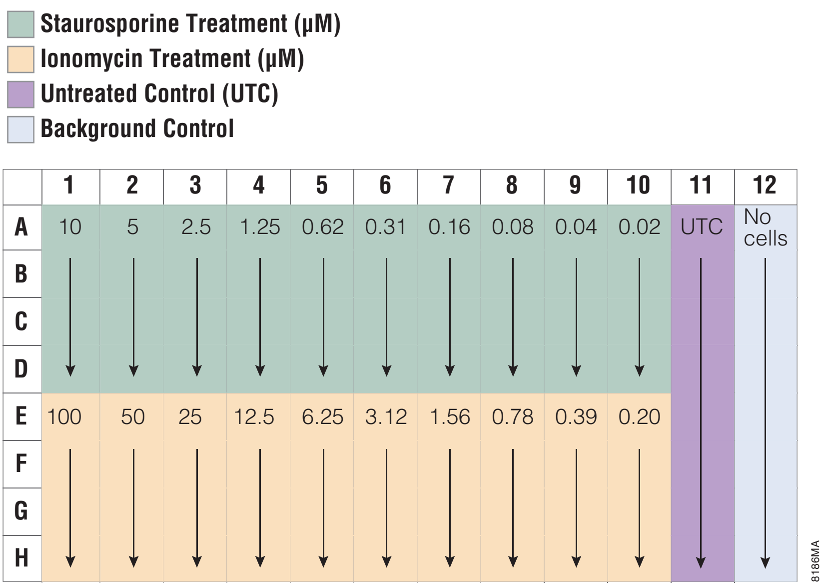

Figure 4. ApoLive-Glo™ Multiplex Assay plate layout following Steps 2–4.

4. Protocol

4.A. Materials to Be Supplied by the User

- 96- or 384-well opaque-walled tissue culture plates with clear or solid bottoms
- multichannel pipette or automated pipetting workstation
- reagent reservoirs
- orbital or linear plate shaker capable of 300–500rpm for 96-well plates or 1,300–1,500rpm for 384-well plates
- microplate reader capable of measuring both luminescence and fluorescence at the following wavelengths:
  excitation ~400nm and emission ~505nm
- positive controls (see Section 3.C for recommendations)

4.B. Example Assay Protocol for 96-Well Plate Format

1. Set up 96-well assay plates containing cells in medium at the selected density.

   Note: We recommend using <20,000 cells per well in a 96-well plate.

2. Add test compounds and vehicle controls to appropriate wells for a final volume of 100µl per well.

   Note: See Section 3.D for an example 96-well plate layout.

3. Culture cells for the desired test exposure period.

   Note: In vitro cytotoxicity is dependent upon compound dosage and cell exposure period. For example, if cells are treated with a slow-acting apoptosis-inducing compound for only 2 hours, it is unlikely that changes in viability or caspase activation will be measurable. Longer exposure times with the same compound will reveal a decrease in viability and an increase in caspase activity. If treated for too long with the compound, the caspase activation window will be missed. If cells are treated with a fast-acting compound for a long exposure period (e.g., 48 hours), viability will be reduced, and there will be no measurable caspase activity. Inappropriate exposures may result in misleading compound profiles. Therefore, we recommend characterizing new compounds in multiple exposure periods (4, 12, 24 and 48 hours) to determine the mechanism of cell death.

4. Add 20µl of Viability Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).

5. Incubate for 30 minutes at 37°C.

   Note: Incubations longer than 30 minutes may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours.

6. Measure fluorescence at the following wavelength set: $400_{Ex}/505_{Em}$.

7. Add 100µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).

4.B. Example Assay Protocol for 96-Well Plate Format (continued)

8. Incubate for 30 minutes at room temperature.

   Note: Incubation times longer than 30 minutes may improve assay sensitivity and dynamic range. See Note 5 in Section 3.A.

9. Measure luminescence.

4.C. Example Assay Protocol for Standard 384-Well Plate Format

1. Set up 384-well assay plates containing cells in medium at the desired density.

   Note: We recommend using <5,000 cells per well in a 384-well plate.

2. Add test compounds and vehicle controls to appropriate wells for a final volume of 20µl per well.

3. Culture cells for the desired test exposure period.

   Note: In vitro cytotoxicity is dependent upon compound dosage and cell exposure period. For example, if cells are treated with a slow-acting apoptosis-inducing compound for only two hours, it is unlikely that changes in viability or caspase activation will be measurable. Longer exposure times with the same compound will reveal a decrease in viability and an increase in caspase activity. If treated for too long with the compound, the caspase activation window will be missed. If cells are treated with a fast-acting compound for a long exposure period (e.g., 48 hours), viability will be reduced, and there will be no measurable caspase activity. Inappropriate exposures may result in misleading compound profiles. Therefore, we recommend characterizing new compounds in multiple exposure periods (4, 12, 24 and 48 hours) to determine the mechanism of cell death.

4. Add 5µl of Viability Reagent to all wells, and briefly mix by orbital shaking (1,300–1,500rpm for ~30 seconds).

5. Incubate for 30 minutes at 37°C.

   Note: Incubations longer than 30 minutes may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours.

6. Measure fluorescence at the following wavelengths: $400_{Ex}/505_{Em}$.

7. Add 25µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (1,300–1,500rpm for ~30 seconds).

8. Incubate for 30 minutes at room temperature.

   Note: Incubation times longer than 30 minutes may improve assay sensitivity and dynamic range. See Note 5 in Section 3.A.

9. Measure luminescence.

5. Example Data

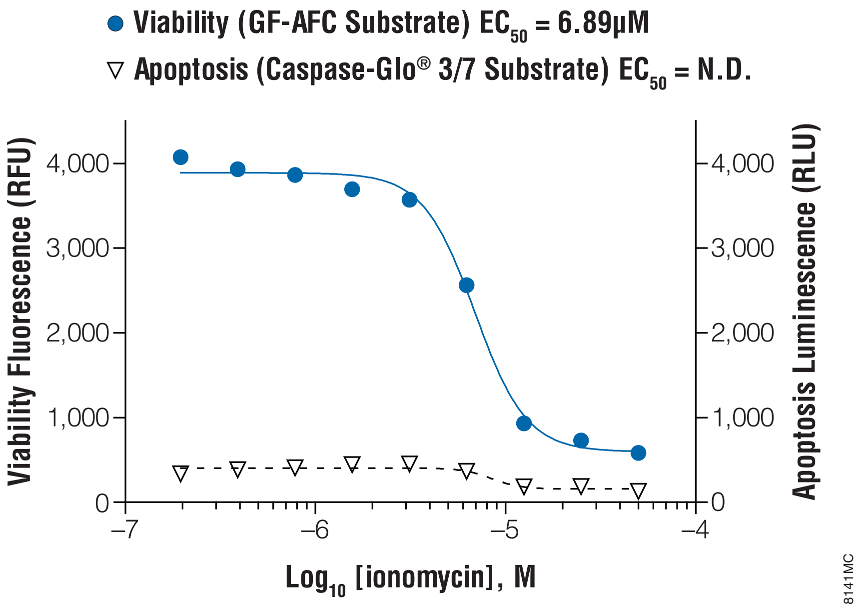

Figure 5. Expected results for ionomycin treatment of Jurkat cells. Ionomycin treatment for 6 hours should result in a dose-dependent decrease in viability with no caspase-3/7 activation, which is consistent with primary necrosis.

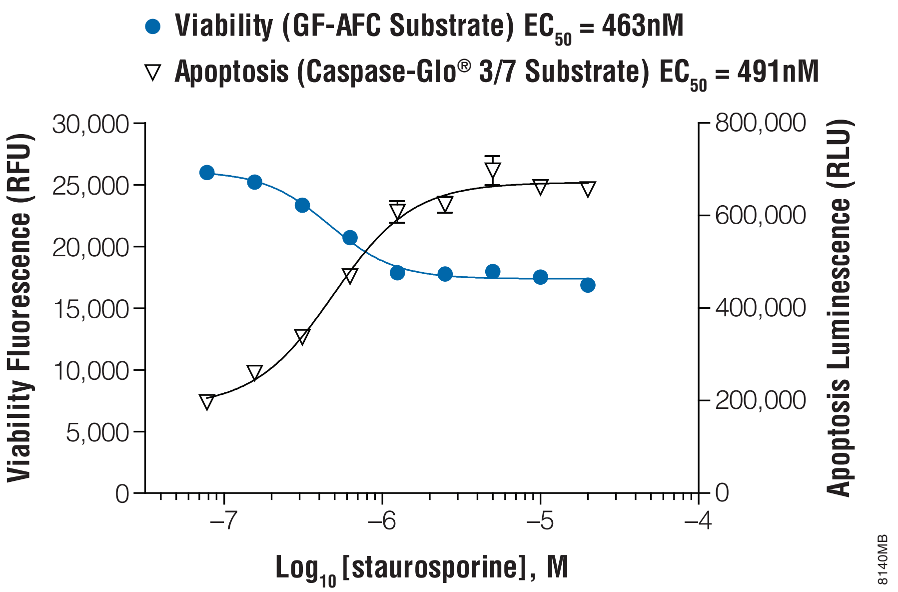

Figure 6. Expected results for staurosporine treatment of Jurkat cells. Staurosporine treatment for 6 hours should result in a dose-dependent decrease in viability and a dose-dependent increase in caspase-3/7 activity consistent with apoptosis.

5. Example Data (continued)

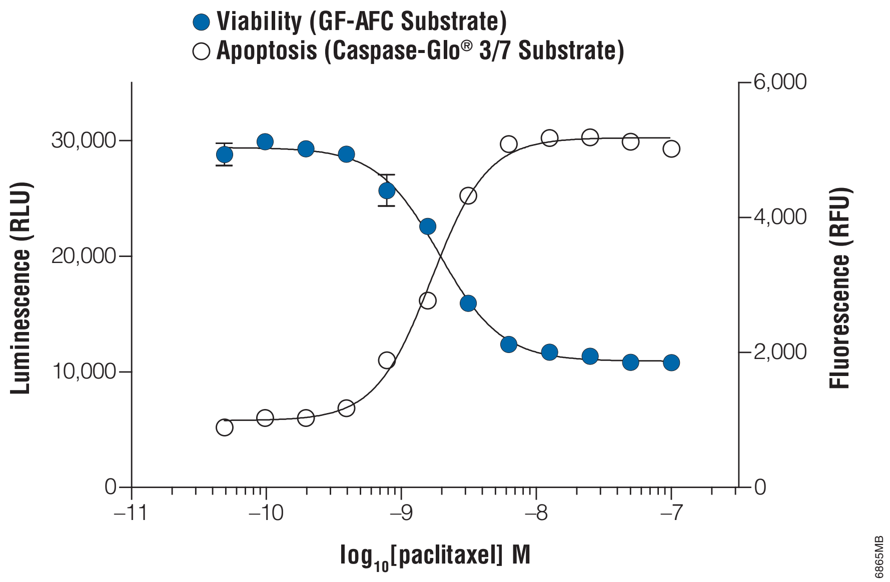

Figure 7. Example assay data from ApoLive-Glo™ Multiplex Assay. The Viability Reagent was added to wells and viability measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent was added and luminescence measured after a 30-minute incubation at room temperature (10,000 Jurkat cells/well in a 96-well plate). Paclitaxel treatment for 24 hours should result in a dose-dependent decrease in cell viability and an increase in caspase-3/7 activity, consistent with apoptosis.

6. General Considerations

This section contains a list of general factors to consider when designing your assay plate layout, interpreting your data accurately and troubleshooting the assay chemistry.

Length of Compound Exposure

The kinetics of cytotoxicity vary among compounds. The caspase biomarkers of apoptosis may degrade in a time-dependent manner. Therefore, consider using this assay at different time points to establish optimal detection of apoptosis. Primary necrosis (or catastrophic cell lysis) tends to occur very quickly after adding a toxic compound (i.e., two hours or less), whereas apoptosis proceeds in a more orderly manner over a longer period (i.e., 4–48 hours).

Selection of Compound Concentration(s)

Consider using serial dilutions of compounds instead of just one concentration in your assay. Many high-throughput screens are performed using a single compound concentration (e.g., 10µM final) to test larger numbers of compounds. However, using only one concentration can be problematic due to factors including biological variation in response and physiochemical concerns such as compound solubility. The approach of quantitative high-throughput screening (qHTS; 5) involves examining each compound in a screen in broad serial-dose dilutions. This approach can be more technically involved but can produce high-quality response curves that allow greater characterization of cytotoxic effects while mitigating false-positive or false-negative test results.

Interpreting the Mechanism of Cell Death

Both assay measures (viability and caspase activation) are important for developing an accurate profile for your compound. It is well appreciated that prototypical anticancer therapeutics may exhibit antiproliferative effects for sustained time periods prior to actual changes in membrane integrity. This period of cell cycle arrest will manifest as an apparent decline in viability. Caspase activation may or may not be measurable during this period. If no caspase activation is detected, primary necrosis or fast-acting apoptosis should be confirmed in a shorter exposure period (6).

Microplate Reader Settings

Fluorescence measurements: Carefully set the excitation and emission settings on your reader (as closely as possible) to: excitation at 400nm / emission at 505nm. Results may suffer if the incorrect settings are selected. See Figure 8 for excitation and emission ranges.

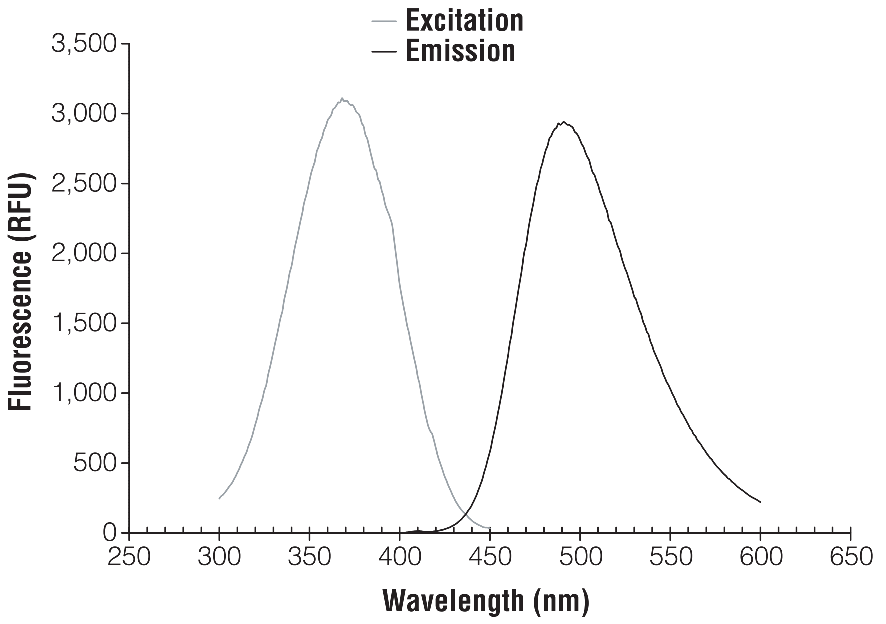

Figure 8. Peak excitation and emission wavelengths for the GF-AFC Substrate.

Luminescence measurements: Confirm that the integration time is set within the following ranges:

- 96-well plates: 0.5–1 second
- 384-well plates: 0.25–0.5 second

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6. General Considerations (continued)

Plotting Data

Consider plotting your data using a log-based transform for the compound concentration. Since the intensity of the fluorescent and luminescent measures (RFU vs. RLU) can differ significantly, consider plotting your data using two Y-axes. Refer to Section 5 for examples.

Multiwell-Plate Selection

Because the ApoLive-Glo™ Multiplex Assay uses both fluorescent and luminescent detection, opaque-walled 96- or 384-well tissue culture plates should be used with the ApoLive-Glo™ Multiplex Assay.

Opaque-walled tissue culture plates are available in several varieties, white or black, with either solid or clear bottoms. The clear-bottom plates offer the advantage of being able to examine the cells by microscopy during the course of the experiment. Either white or black plates can be used with the ApoLive-Glo™ Multiplex Assay. The primary difference between white and black plates is their reflective properties. White plates reflect light and will maximize light output signal; black plates absorb light and reduce background and crosstalk. For these reasons, white plates are commonly used for luminescent assays and black plates are used for fluorescent assays. When multiplexing a luminescent and fluorescent assay, the use of a white plate would support maximum light output signal for the luminescent portion of the assay but result in higher crosstalk and background for fluorescence. The use of a black plate in a multiplex assay would reduce fluorescent signal crosstalk and background with a reduction in the luminescent signal.

Several examples of 96-well plates appropriate for use with the ApoLive-Glo™ Multiplex Assay are listed below:

solid-bottom white plates
- Corning Costar® Cat.# 3917
- Greiner Bio-One CELLSTAR Cat.# 655073

clear-bottom white plates
- BD Biosciences Optilux™ Cat.# 353947
- Corning Costar® Cat.# 3903
- Greiner Bio-One CELLSTAR Cat.# 655088

solid-bottom black plates
- Corning Costar® Cat.# 3916
- Greiner Bio-One CELLSTAR Cat.# 655079
- Nunc™ F96 MicroWell™ Plates Cat.# 137101

clear-bottom black plates
- Corning Costar® Cat.# 3904
- Greiner Bio-One CELLSTAR Cat.# 655087

Other Factors

- Some compounds or cell culture medium components or both can influence the assay measures due to factors such as native background fluorescence.
- Significant temperature fluctuations during the assay may affect assay performance.
- Minimize the amount of compound carrier (i.e., %DMSO) in the assay.

For additional information, see the General Considerations sections of the *CellTiter-Fluor™ Cell Viability Assay Technical Bulletin* #TB371 and the *Caspase-Glo® 3/7 Assay Technical Bulletin* #TB323, available online at: **www.promega.com/protocols/**

7. Literature Cited

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8. Additional Resources

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8. Additional Resources (continued)

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9. Related Products

Multiplexed Viability, Cytotoxicity and Apoptosis Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml	G6320
	5 × 10ml	G6321
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml*	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml*	G9200

*Available in additional sizes.

Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

Available in additional sizes.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 2 Assay*	10ml	G0940
Caspase-Glo® 3/7 Assay*	10ml	G8091
Caspase-Glo® 6 Assay*	10ml	G0970
Caspase-Glo® 8 Assay*	10ml	G8201
Caspase-Glo® 9 Assay*	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790

Available in additional sizes.

Cytotoxicity Assays

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260

Available in additional sizes.

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911

Available in additional sizes

Detection Instrumentation

Product	Size	Cat.#
GloMax®-Multi Detection System Base Instrument	each	E7031
GloMax®-Multi+ Detection System Base Instrument with Shaking	each	E8031

[a]U.S. Pat. Nos. 7,416,854, 7,553,632 and other patents pending.

[b]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294 and other patents and patents pending.

[c]U.S. Pat. Nos. 7,148,030, 7,384,758, Australian Pat. No. 2003216139 and other patents pending.

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

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