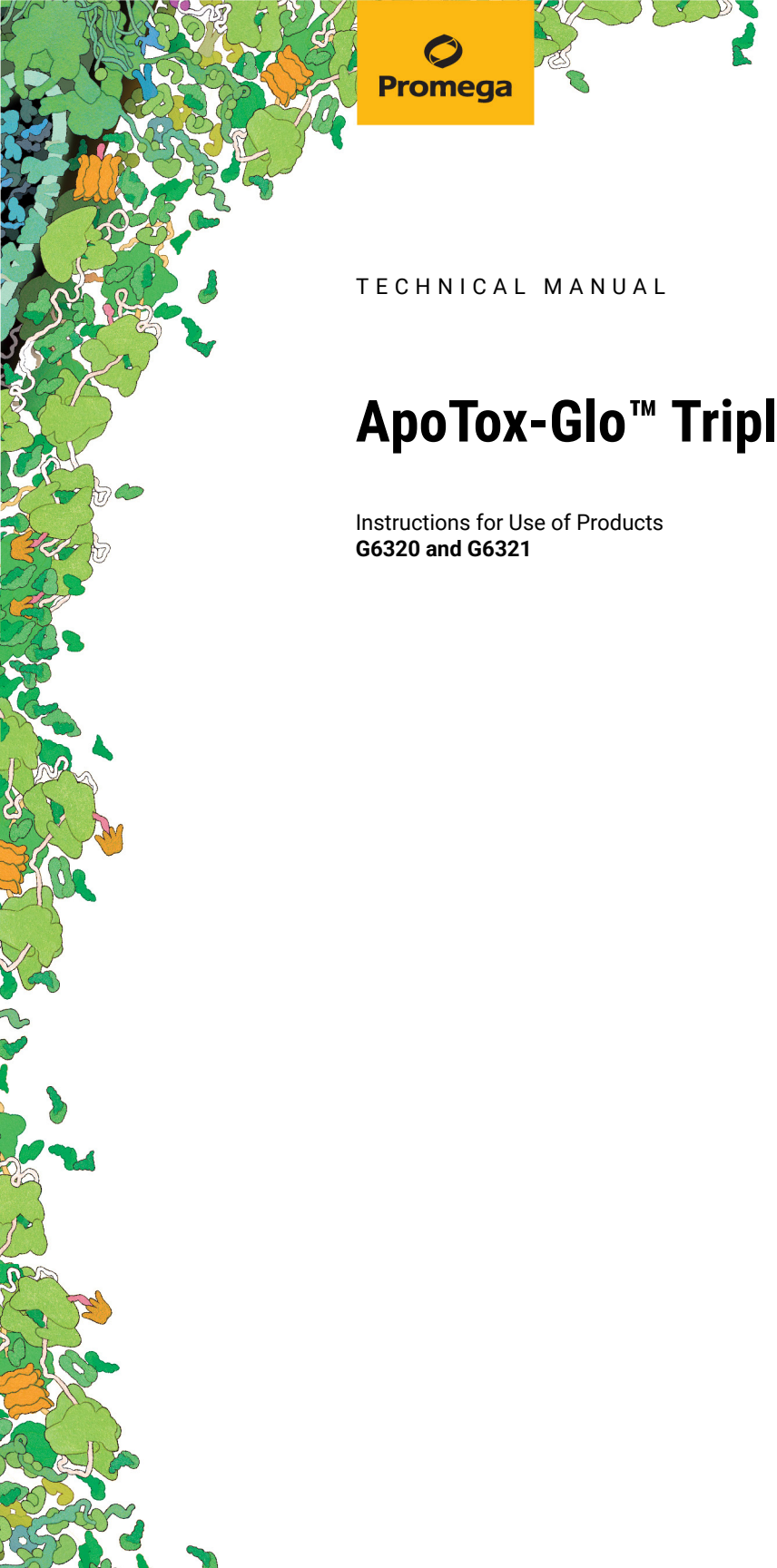

TECHNICAL MANUAL

# ApoTox-Glo™ Triplex Assay

Instructions for Use of Products
**G6320 and G6321**

Revised 11/26
TM322

Promega

# ApoTox-Glo™ Triplex Assay

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


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## 1. Description

The ApoTox-Glo™ Triplex Assay combines three Promega assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. The first part of the assay simultaneously measures two protease activities; one is cathepsin C, a marker of cell viability, and the other is tripeptidyl peptidase II, a marker of cytotoxicity (1). The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenyl-alanyl-aminofluorocoumarin; 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. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity (Figure 1). Because bis-AAF-R110 is not cell-permeant, essentially no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously (2).

## 1. Description (continued)

The second part of the assay uses the Caspase-Glo® Assay Technology by providing 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; 3). 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.

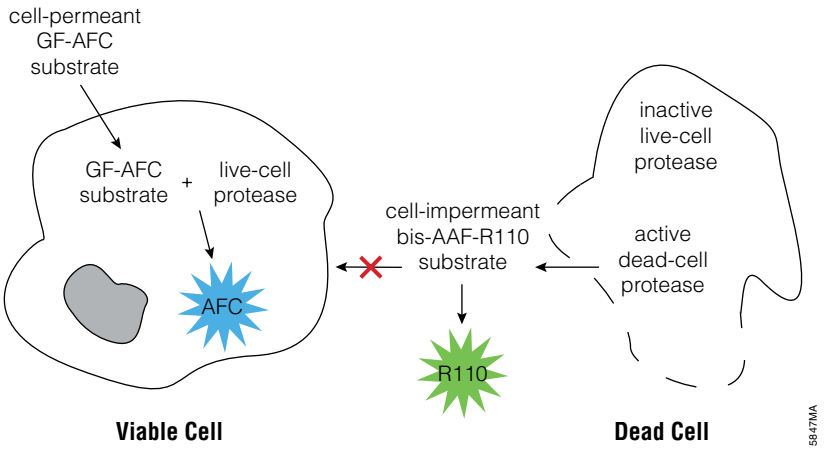


**Figure 1. The biology of the viability/cytotoxicity assay.** The GF-AFC Substrate can enter live cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 Substrate cannot enter live cells but instead can be cleaved by the dead-cell protease to release R110.

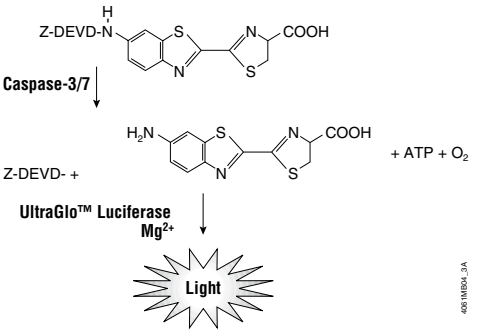


**Figure 2. Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence.** Following caspase cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase reaction and the production of light.

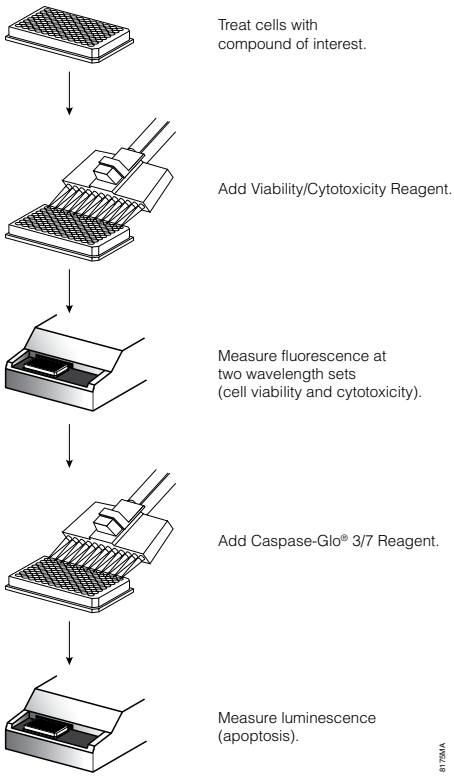


**Figure 3. Schematic diagram of the ApoTox-Glo™ Triplex Assay.** Cell viability fluorescence is measured at $400_{Ex}/505_{Em}$, cytotoxicity fluorescence is measured at $485_{Ex}/520_{Em,}$ while apoptosis is determined with the luminescence measurement.

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## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **ApoTox-Glo™ Triplex Assay** | **10ml** | **G6320** |

For in vitro use only. Cat.# G6320 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 × 10µl bis-AAF-R110 Substrate (100mM in DMSO)
- 1 × 10ml Caspase-Glo® 3/7 Buffer
- 1 bottle Caspase-Glo® 3/7 Substrate (lyophilized)

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **ApoTox-Glo™ Triplex Assay** | **5 × 10ml** | **G6321** |

For in vitro use only. Cat.# G6321 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)
- 1 × 50µl bis-AAF-R110 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 –30°C to –10°C protected from light. See product label for expiration date information.

## 3. Reagent Preparation and Storage of Prepared Reagents

### Reagent Preparation

1. Thaw each assay component as follows:
   **Assay Buffer:** 37°C water bath
   **GF-AFC Substrate:** 37°C water bath
   **bis-AAF-R110 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 and bis-AAF-R110 Substrate into 2.0 or 2.5ml of Assay Buffer, depending on the plate format used. For 96-well plates, transfer 10µl of each substrate into 2ml of Assay Buffer. For standard 384-well plates, transfer 10µl of each substrate into 2.5ml of Assay Buffer. Mix the Assay Buffer containing substrates by vortexing the contents until the substrates are thoroughly dissolved. This mixture will be referred to as the Viability/Cytotoxicity Reagent.

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

3. Transfer the contents of the Caspase-Glo® 3/7 Buffer bottle into the 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<br>Stored for 1 week = ~90% signal<br>Stored for 4 weeks = ~75% signal |
| –20°C | Stored up to 1 week = ~75% signal<br>Stored up to 4 weeks = ~60% signal |

## 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 sets of wavelengths:
  Excitation ~400nm and Emission ~505nm
  Excitation ~485nm and Emission ~520nm
- positive controls (see Section 4.E for recommendations)

### 4.B. Before You Begin

Before starting the assay, prepare the Assay Buffer with both substrates and Caspase-Glo® 3/7 Reagent as directed in Section 3. Because of the sensitivity of this assay, be careful not to touch pipette tips to the wells containing samples to avoid cross-contamination. Between dispensings, cover the plate with a lid or plate seal to minimize exposure to contaminants in the air. If you are reusing pipette tips, do not touch pipette tips to the wells containing samples to avoid cross-contamination.

**Note:** Temperature fluctuations can affect the luminescence readings. If the room temperature fluctuates, use a constant-temperature incubator. 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.

### 4.C. 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 4.E 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. The kinetics of measurable cytotoxic biomarkers can vary widely between individual compounds and treatments. For example, if cells are treated with a slow-acting apoptosis-inducing compound for only 2 hours, it is unlikely that changes in viability, cytotoxicity or caspase activation will be measurable. Longer exposure times with the same compound will reveal cytotoxicity. Alternatively, if cells are treated with a fast-acting compound for a long exposure period (e.g., 48 hours), viability will be reduced, but cytotoxicity may be underestimated. Inappropriate exposures may result in misleading compound profiles. Therefore, it is important to characterize new compounds in multiple exposure periods (4, 12, 24 and 48 hours).

4. Add 20µl of Viability/Cytotoxicity Reagent containing both GF-AFC Substrate and bis-AAF-R110 Substrate 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 two wavelength sets:
   $400_{Ex}/505_{Em}$ (Viability)
   $485_{Ex}/520_{Em}$ (Cytotoxicity)

7. Add 100µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (300–500rpm 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 in Section 4.B.

9. Measure luminescence (caspase activation, a hallmark of apoptosis).

### 4.D. 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.

   **Note:** See Section 4.E 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. The kinetics of measurable cytotoxic biomarkers can vary widely between individual compounds and treatments. For example, if cells are treated with a slow-acting apoptosis-inducing compound for only 2 hours, it is unlikely that changes in viability, cytotoxicity or caspase activation will be measurable. Longer exposure times with the same compound will reveal cytotoxicity. Alternatively, if cells are treated with a fast-acting compound for a long exposure period (e.g., 48 hours), viability will be reduced, but cytotoxicity may be underestimated. Inappropriate exposures may lead to misleading profiles. Therefore, it is important to characterize new compounds in multiple exposure periods (4, 12, 24 and 48 hours).

4. Add 5µl of Viability/Cytotoxicity Reagent containing both GF-AFC Substrate and bis-AAF-R110 Substrate 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 two wavelength sets:
   $400_{Ex}/505_{Em}$ (Viability)
   $485_{Ex}/520_{Em}$ (Cytotoxicity)

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 in Section 4.B.

9. Measure luminescence (caspase activation, a hallmark of apoptosis).

### 4.E. 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.

**Positive Controls**

**Cell Viability and Cytotoxicity:** 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., 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., 10µM staurosporine for 6 hours).

**Note:** It is important to use identical cell numbers and volumes for the assay and the control samples. You may need to empirically determine the optimal cell number, apoptosis induction treatment and incubation time for the 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.

**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. See Figure 4 for plate layout.
2. Add 50µl of RPMI 1640 + 10% FBS to columns 2–12 of a 96-well assay plate.
3. Add 50µl of control compound to replicate wells in columns 1 and 2. Mix the contents of column 2 by pipetting and transfer to column 3.
4. Repeat the mixing and transfer of compound until column 10. Discard the 50µl removed from column 10. This creates twofold serial dilutions from column 1 through column 10.
5. Prepare Jurkat cells at a concentration of 200,000 cells/ml, and dispense 50µl (a total of 10,000 cells) to all wells except column 12.
6. Add 50µl of medium and vehicle to column 12. Final volume in all wells will be 100µl.
7. Incubate the cells for 6 hours at 37ºC.
8. Add 20µl of Viability/Cytotoxicity Reagent containing 10µl of each substrate in 2ml of Assay Buffer to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).
9. Incubate for at least 30 minutes at 37°C.

10. Measure fluorescence at the following two wavelength sets:
    $400_{Ex}/505_{Em}$ (Viability)
    $485_{Ex}/520_{Em}$ (Cytotoxicity)
11. Add 100µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).
12. Incubate for 30 minutes at room temperature.
13. Measure luminescence (apoptosis).

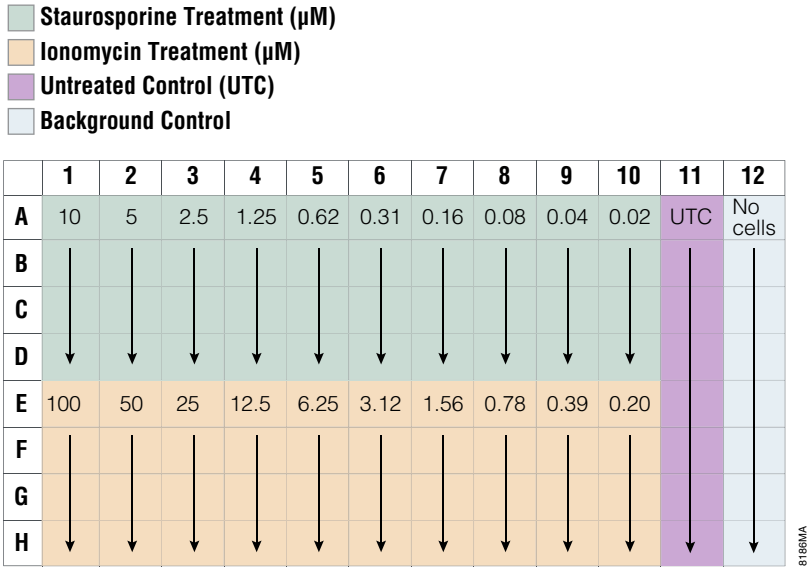


**Figure 4. ApoTox-Glo™ Assay plate layout following Steps 2–6.**

### 4.E. Recommended Controls (continued)

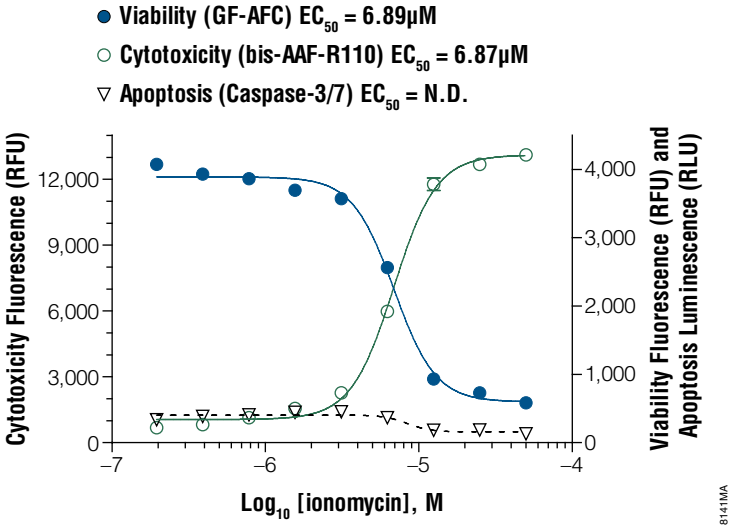


**Figure 5. Expected results for ionomycin treatment of Jurkat cells.** Ionomycin treatment for 6 hours should result in a dose-dependent decrease in viability, increase in cytotoxicity with no caspase-3/7 activation, which is consistent with primary necrosis.

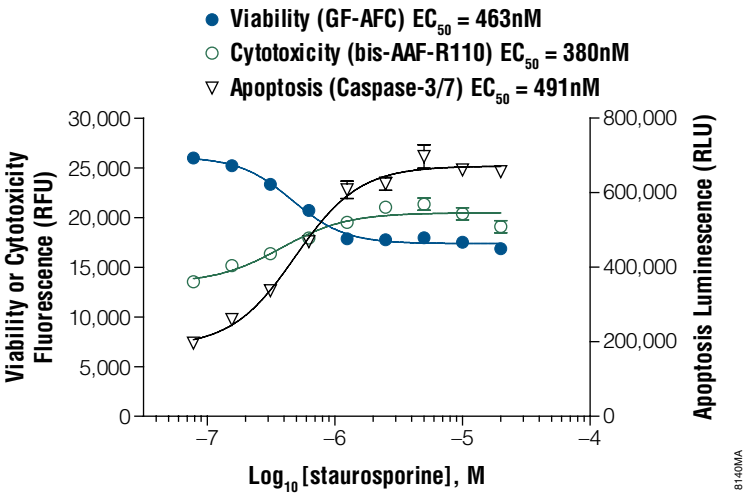


**Figure 6. Expected results for staurosporine treatment of Jurkat cells.** Staurosporine treatment for 6 hours should result in a dose-dependent decrease in viability, increase in cytotoxicity with an increase in caspase-3/7 activity consistent with apoptosis.

## 5. 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 biomarkers of cytotoxicity and apoptosis may degrade in a time-dependent manner. Therefore, consider using this assay at different time points to establish optimal detection of cytotoxic affects or apoptosis. Primary necrosis (or catastrophic cell lysis) tends to occur very quickly after adding a toxic compound (i.e., 2 hours or less), whereas apoptosis proceeds in a more orderly manner over a longer period (i.e., 4–48 hours).

During most cytotoxicity events, viability and cytotoxicity measures will be inversely proportional. That is, if viability assay relative fluorescent units (RFU) are high, then cytotoxicity assay RFU values will be low, or vice versa. However, depending on the length of compound exposure, this inverse relationship does not always hold true. For example, after long-term exposure >24 hours, particularly after early primary necrosis occurs, the cytotoxicity biomarker will degrade after release into the extracellular environment and may lead to an underestimation of cytotoxicity. A reduction in viability without an increase in cytotoxicity might also be seen with compounds that alter normal cell division (cell-cycle arrest) without producing membrane integrity changes (Figure 7).

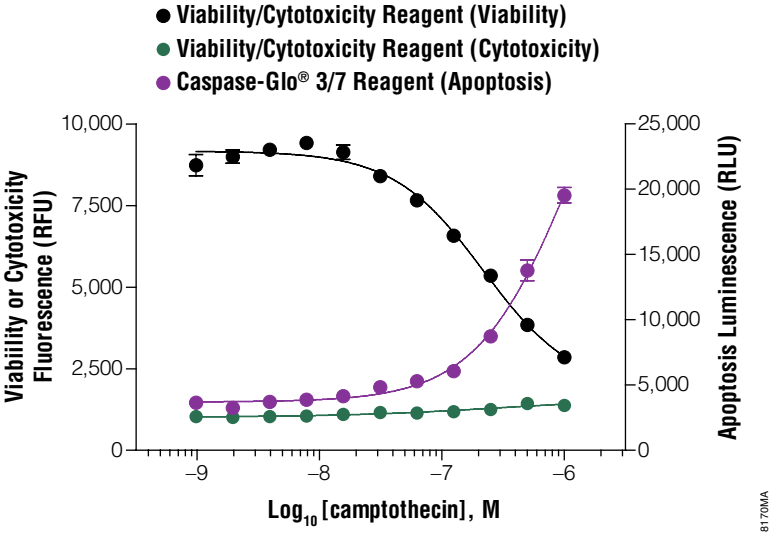


**Figure 7. An example of cytostasis seen using the ApoTox-Glo™ Triplex Assay.** Camptothecin treatment of 10,000 K562 human erythroleukemia cells for 48 hours resulted in a dose-dependent decrease in apparent viability with no cytotoxicity, but an increase in caspase-3/7 activity, a profile consistent with cell cycle arrest and early phase apoptosis.

## 5. General Considerations (continued)

### 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; 4) 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

All three assay measures (viability, cytotoxicity, and caspase activation) are important for developing an accurate profile for your compound. In most circumstances, viability and cytotoxicity will be inversely correlated. However, 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 with no concomitant increase in cytotoxic biomarker. Caspase activation may or may not be measurable during this period. Conversely, a measurable decline in apparent viability may be paired with a substantially reduced or unmeasurable cytotoxicity biomarker if cells died early (typically by primary necrosis) in the exposure period. If no caspase activation is indicated, primary necrosis or fast-acting apoptosis should be confirmed in a shorter exposure period (5).

### Microplate Reader Settings

**Fluorescent measurements:** Carefully set the excitation and emission settings on your reader (as closely as possible) as follows:
**Viability:** Excitation at 400nm / Emission at 505nm
**Cytotoxicity:** Excitation at 485nm / Emission at 520nm

Results may suffer if the incorrect settings are selected. See Figure 8 for excitation and emission ranges.

**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

### Plotting Data

Consider plotting your data using a log-based transform for the compound concentration. Because 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 4.E for examples.

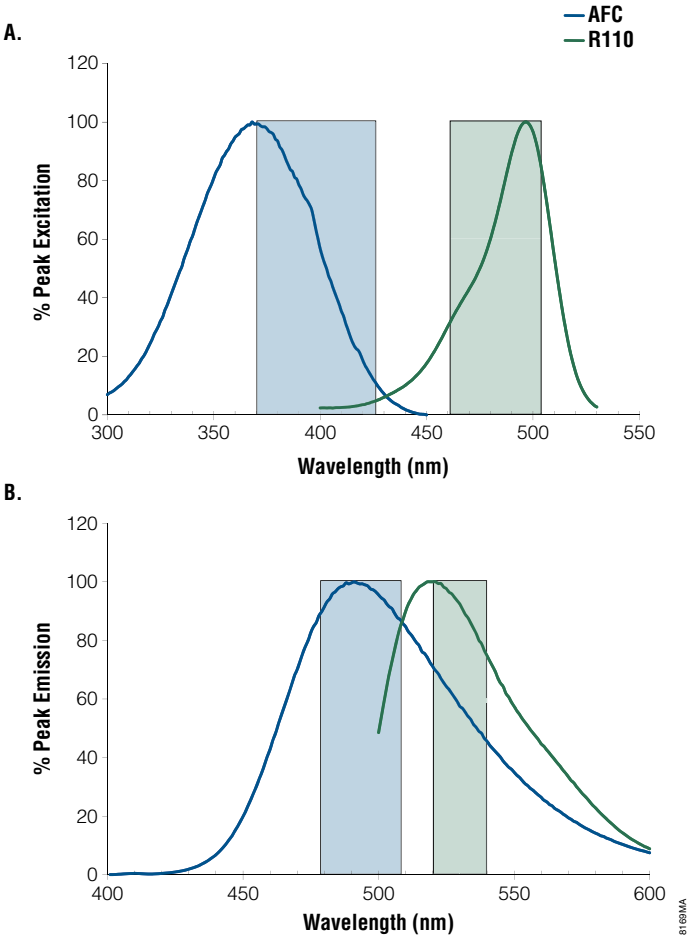


**Figure 8. Peak excitation and emission wavelengths for the GF-AFC and bis-AAF-R110 Substrates.** Boxes indicate the optimal filter sets ranges required for separating and measuring the fluorescence signals.

**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 *MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin* #TB348 and the *Caspase-Glo® 3/7 Assay Technical Bulletin* #TB323, available online at: **www.promega.com/protocols/**

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## 8. Summary of Changes

The following changes were made to the 11/26 revision of this document:

1. Updated Sections 1 and 6.
2. Removed the Related Products section.
3. Updated the cover page and document font.
4. Removed patent statements.
5. Made minor text edits.

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