# MEASURE RELATIVE NUMBERS OF LIVE AND DEAD CELLS AND NORMALIZE ASSAY DATA TO CELL NUMBER

ANDREW NILES[1], MICHAEL SCURRIA[2], LAURENT BERNAD[2], BRIAN MCNAMARA[1], KAY RASHKA[1], DEBORAH LANGE[1], PAM GUTHMILLER[1], TRACY WORZELLA[1] AND TERRY RISS[1], [1]PROMEGA CORPORATION, [2]PROMEGA BIOSCIENCES

Here we elaborate on distinguishing features of the MultiTox-Fluor Multiplex Cytotoxicity Assay (1–3) and introduce the luminescent, next-generation technology: The CytoTox-Glo™ and MultiTox-Glo Multiplex Cytotoxicity Assays.

## Introduction

Experimental cell biology continues to use an assortment of mammalian cells in a multitude of in vitro response models. Primary, transformed, or transfected cells are cultured in the wells of an assay plate and treated with chemical, physical or biological stimuli. The motivation for using these "cause-and-effect" models ranges from identifying critical pathway checkpoints and modulators of molecular processes within a cellular context to testing the predictive cytotoxic potential of the treatment. Nevertheless, because cellular responses are intrinsically linked to viability and cytotoxicity, researchers need to measure cellular health after experimental manipulation.



Many methods exist to determine viability or cytotoxicity in cell-based models (4). Most viability or cytotoxicity assays directly or indirectly measure the presence or absence of a functional cellular membrane as a barrier to the extracellular environment. Here we describe the inherent utility and multifunctionality of the CytoTox-Glo™, MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assay[(a)] technologies for measuring the relative number of both live and dead cells in culture wells. These assays can deliver ratiometric, inversely correlated values of viability and cytotoxicity. These ratiometric values are useful for normalizing data to cell number and reduce incorrect interpretation of data due to assay interferences. Lastly, depending on the assay and configuration, these reagents are compatible with additional fluorescent and luminescent multiplexed chemistries.

## Assay Principles and Chemistries

The MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays measure the same two distinct protease activities as markers for cell viability or cytotoxicity but offer slightly different detection formats, fluorescence or luminescence (Figures 1 and 2). After incubation, the fluorescent products of the assays are conveniently measured using standard fluorometry. The luminescent signal is measured using a multimode instrument or luminometer.

The CytoTox-Glo™ Cytotoxicity Assay uses the AAF-Glo™ chemistry to measure the relative number of dead cells as a luminescent signal. However, because of the stability of the signal achieved at the luminescence reaction steady-state, a lysis solution can be added (after cytotoxicity values are

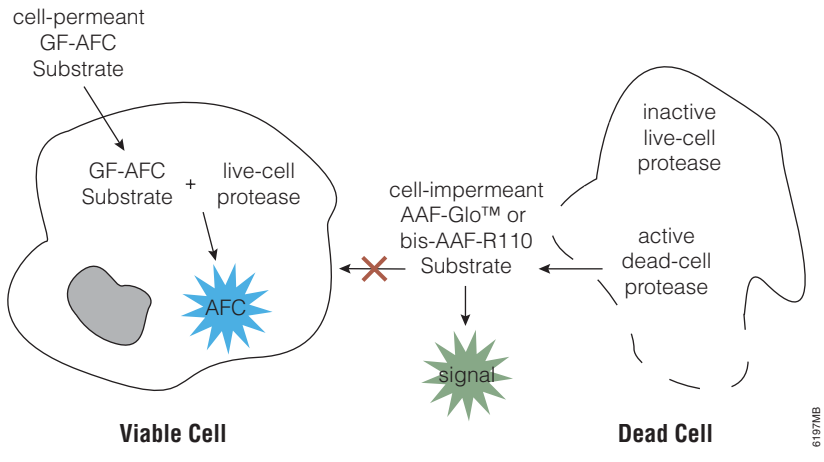



**Figure 1. The assay principle and chemistry behind the MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays.** The live-cell protease activity is measured in both assay formats by the fluorogenic, cell-permeant, peptide substrate Gly-Phe-7-amino-4 trifluoromethyl coumarin (GF-AFC). This live-cell protease activity marker becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium and does not contribute to the live-cell response. The second, dead-cell protease activity marker is measured from cells that have lost membrane integrity. In the MultiTox-Fluor Multiplex Cytotoxicity Assay, this activity is measured with the cell-impermeant, fluorogenic peptide substrate, bis-(Ala-Ala-Phe)-rhodamine 110 (bis-AAF-R110). The MultiTox-Glo Multiplex Cytotoxicity Assay measures this dead-cell protease activity with the cell-impermeant luminogenic substrate, Ala-Ala-Phe-aminoluciferin (AAF-Glo™ Substrate).

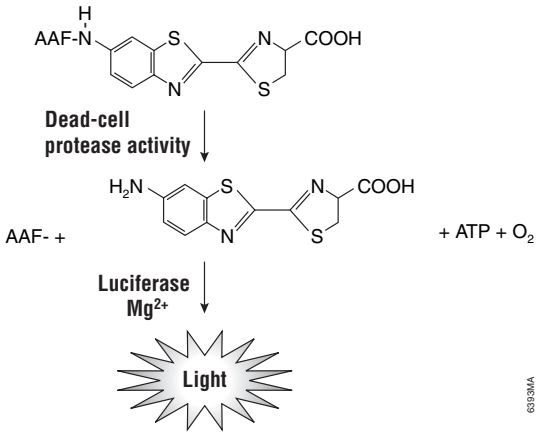



**Figure 2. Cleavage of the luminogenic AAF-Glo™ Substrate by dead-cell protease activity in the presence of luciferase, $Mg^{2+}$ and ATP provided in the reagent formulation.** Following cleavage, a substrate for luciferase (aminoluciferin) is released, resulting in the luciferase-mediated production of light.

measured) to detect the relative number of viable cells remaining in the wells. This "viability" value is obtained by subtracting the experimental dead-cell luminescence RLU value from the total dead-cell protease RLU value (cells that died from experimental treatment plus viable cells lysed by detergent).

## Ratiometric Response Allows You to Normalize Data

The markers for viability and cytotoxicity in the MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays are independent proteolytic responses but are inversely correlated and complimentary as long as the half-life of the protease is not limiting (Figure 3, Panels A and B). Although only one marker is used in the CytoTox-Glo™ Cytotoxicity Assay, the responses are ratiometrically linked because they reflect the number of dead and viable cells (after reagent lysis) after experimental treatment (Figure 3, Panel C). This allows you to normalize assay data to cell number and mitigate assay interferences that may lead to erroneous conclusions.

## Benefit from a Longer Protease Half-Life

All enzymatic markers for cytotoxicity have finite activity half-lives. The protease markers in these assays have improved stability over those in other luciferase-based cytotoxicity assays (Figure 4). However, exposing cells to test compounds for more than 24 hours can result in underestimated cytotoxicity. If exposures longer than 24 hours are required, reducing the initial compound concentration may influence the kinetics of cell death and allow you to better assess cytotoxicity.

## Collect High-Quality Data Using This Sensitive Chemistry

The practical utility and sensitivity of any detection chemistry is defined by the total number of cells required ("signal window"), the assay's ability to discriminate between low numbers of treated and untreated cells (sensitivity and variation), and assay interferences (background signals) associated with cells or medium components. The CytoTox-Glo™, MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays are sensitive and scalable in a variety of plate density formats (96-, 384- and 1536-well). The utility of

Table 1. Choosing the Cytotoxicity Assay that Works for You.

| | Live-Cell Protease Marker | Dead-Cell Protease Marker | Sensitivity | Features | Can Be Performed in Multiplex with: |
|---|---|---|---|---|---|
| MultiTox-Fluor Multiplex Cytotoxicity Assay (Cat.# G9200, G9201, G9202) | GF-AFC (fluorescent) | bis-AAF-R110 (fluorescent) | can detect changes in viability as small as 2–5% after 30-minute incubation | ratiometric measures allow well-to-well normalization of data; two independent channels allow you to flag assay interferences | Caspase-Glo® 3/7, 8 and 9 Assays; CellTiter-Glo® Assay and luminescent reporter assays |
| CytoTox-Fluor™ Cytotoxicity Assay[a] (Cat.# G9260, G9261, G9262) | Not applicable | bis-AAF-R110 (fluorescent) | can detect changes in viability as small as 2–5% after 30-minute incubation | compatible with spectrally distinct downstream assays with no color quenching | Caspase-Glo® 3/7, 8 and 9 Assays; CellTiter-Glo® Assay and luminescent reporter assays |
| MultiTox-Glo Multiplex Cytotoxicity Assay (Cat.# G9270, G9271, G9272) | GF-AFC (fluorescent) | AAF-aminoluciferin (luminescent) | can detect changes in viability as small as 2–5% after 15- or 30-minute incubation | ratiometric measures allow well-to-well normalization of data; two independent channels allow you to flag assay interferences | |
| CytoTox-Glo™ Cytotoxicity Assay (Cat.# G9290, G9291, G9292) | optional total lysis protocol in Technical Bulletin #TB359. | AAF-aminoluciferin (luminescent) | can detect changes in viability as small as 2–5% after 15-minute incubation | ratiometric measures allow well-to-well normalization when total lysis protocol is used; no fluorescence interference | |

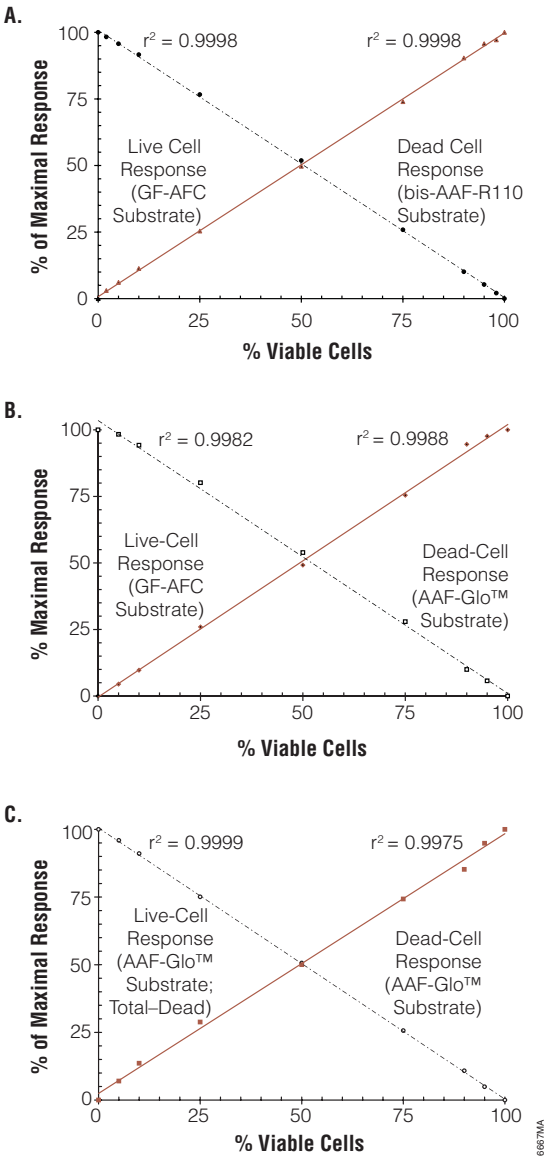



**Figure 3. Ratiometric responses obtained from the CytoTox-Glo™, MultiTox-Fluor and MultiTox-Glo Assays.** A pool of Jurkat cells was adjusted to 100,000 cells/ml and divided into two fractions. One fraction was compromised by treatment to induce cytotoxicity. The other fraction was left untreated. The two fractions were then combined in various proportions to represent varying viabilities from 0–100%. Ten thousand cell equivalents were added to each well in 100µl volumes. Assay reagents were added as directed in the accompanying Technical Bulletins and signals collected using a BMG POLARstar multimode plate reader. The data for the live- and dead-cell responses were normalized to the percentage of the maximal response (See Technical Bulletin #TB359 for total lysis protocol.). **Panel A**. MultiTox-Fluor Assay. **Panel B.** MultiTox-Glo Assay. **Panel C.** CytoTox-Glo™ Assay (with lysis procedure).

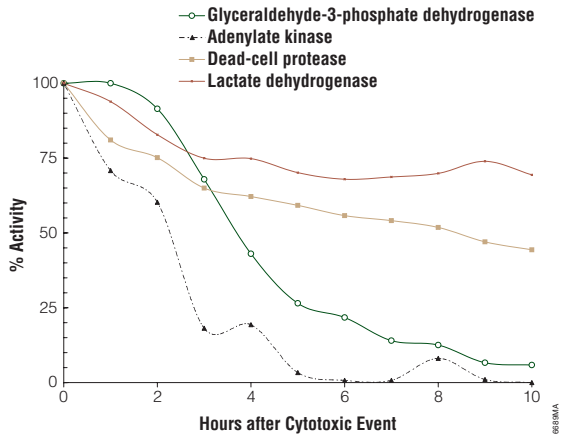



**Figure 4. Half-life of enzymatic markers for cytotoxicity.** Jurkat cells were plated in white-walled plates in 100µl volumes at 10,000 cells/well in RPMI 1640 medium + 10% FBS. Digitonin was added to appropriate replicate wells in 10µl volumes (30µg/ml final concentration) every hour for 10 hours to achieve maximal cytotoxicity. The plate was incubated at 37ºC during this period. CytoTox-Glo™, aCella™-Tox (CellTechnology) and Toxi-Light™ (Cambrex) reagents were prepared and added as directed. Luminescence was measured after 15 minutes using a BMG POLARstar luminometer. CytoTox-ONE™ Reagent was prepared and added as directed. Fluorescence was measured using a Fluoroskan Ascent reader. All data were background substracted and plotted as a percentage of the last lysis time point.

the assays in high-throughput formats was demonstrated by the high Z´-factors achieved in two different cytotoxicity models using automated dispensing systems (Z´-factors ≥ 7.5; Figure 5). Z´-factors greater than 0.5 are considered to be "excellent" assays (5).

## Get Reliable Results and Easily Identify Problematic Data

The CytoTox-Glo™, MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays use novel methods to measure protease activities to determine viability and cytotoxicity of cells in culture. However, because the technology measures distinct changes in membrane integrity, data derived from the assays correlate well with existing methodologies. Therefore, these proteolytic measures deliver data substantially similar to that obtained from resazurin reduction (CellTiter-Blue® Assay), lactate dehydrogenase release (CytoTox-ONE™ Assay ), or ATP detection chemistries (CellTiter-Glo® Assay; Figure 6). Again, because of the ratiometric relationship between live and dead cells using these assays when protease half-life is not limiting, there is also good agreement in the $EC_{50}$ values obtained in separate channels by different proteases (or by the same protease when using the lytic technique described for the CytoTox-Glo™ Assay). The ability to collect ratiometric $EC_{50}$ values has particular merit when conducting compound screening by multipoint testing. Equivalent values can increase the confidence in a potency value, whereas poor agreement can suggest assay interferences or cytostatic

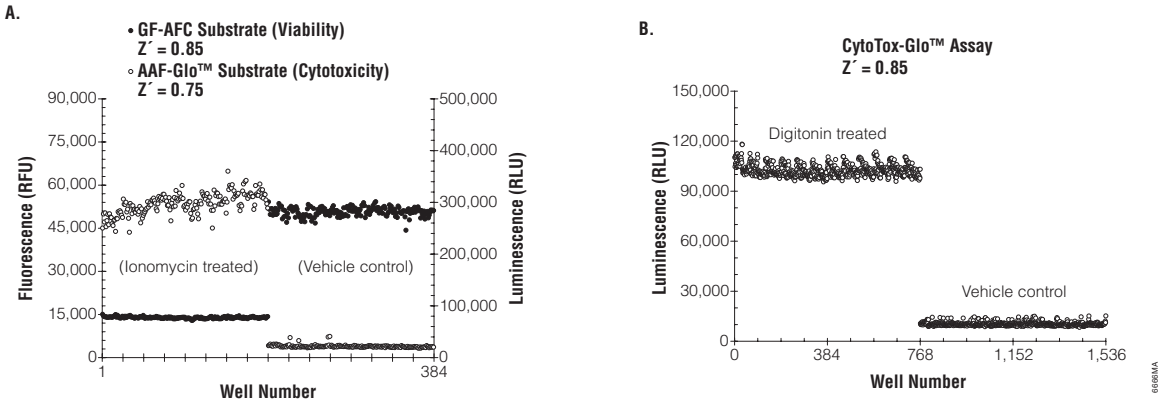
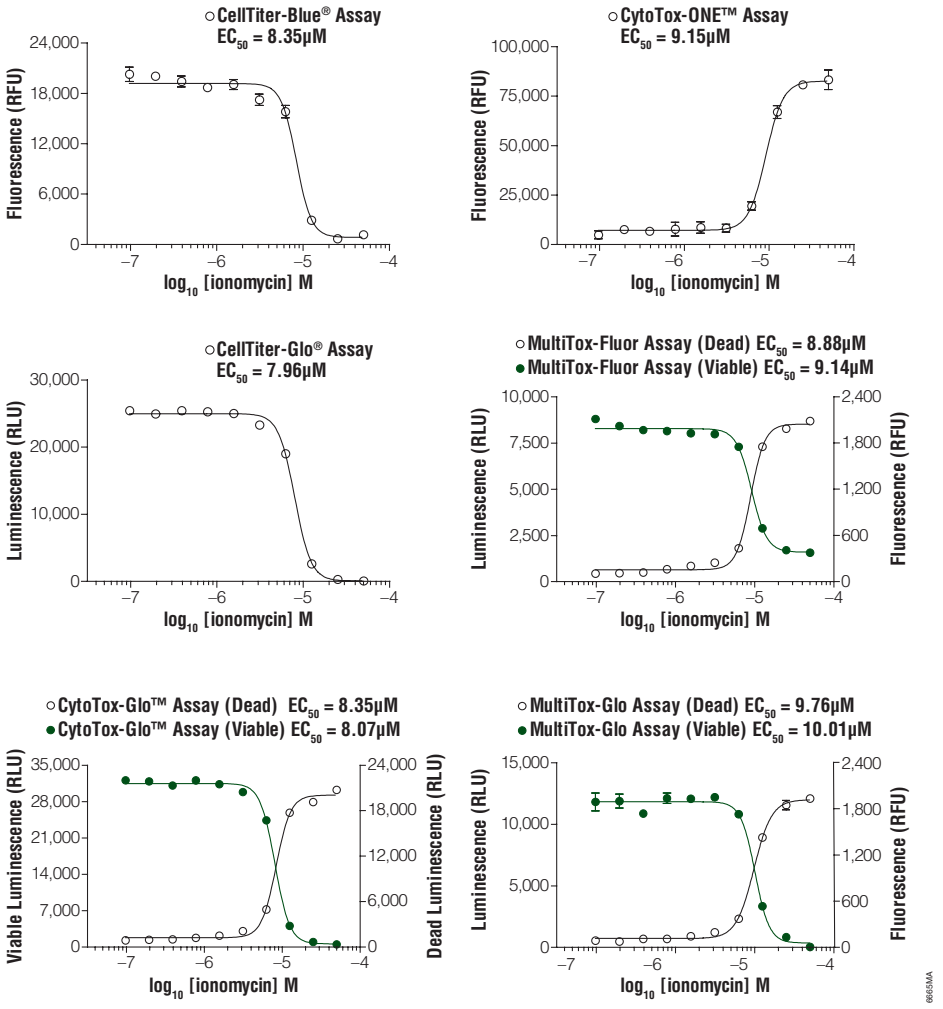



**Figure 5. High assay sensitivities translate into high Z´-factors. Panel A.** Five thousand cells per well were delivered to a 384-well plate in 10µl volumes with the CyBio CyBi®-Well 384/1536 pipettor. Ionomycin (50µM) or vehicle control was added and incubated at 37°C for 2 hours. MultiTox-Glo reagents were added as described in Technical Bulletin #TB358. Fluorescence and luminescence were measured using the Tecan Safire2™ plate reader. **Panel B.** Cells were adjusted to 6.25 × $10^5$ cells/ml, and the pool of cells was split. One fraction was treated by digitonin lysis to simulate cytotoxicity. The other was untreated. The cell equivalents were delivered using the CyBi®-Well pipettor in 4µl volumes. CytoTox-Glo™ Reagent was delivered using the CyBi®-Well pipettor, and luminescence was measured using the Tecan Safire2™ plate reader.

**Figure 6. Concordance with conventional viability and cytotoxicity measures.** Jurkat cells were seeded at a density of 10,000 cells per well in 96-well plates. Serial doses of ionomycin were added to wells and incubated for 6 hours at 37°C. CellTiter-Blue®, CytoTox-ONE™, CellTiter-Glo®, MultiTox-Fluor, CytoTox-Glo™ and MultiTox-Glo Reagents were prepared and added, and signals were measured as described in the technical literature. Potency values were determined using GraphPad Prism® software.



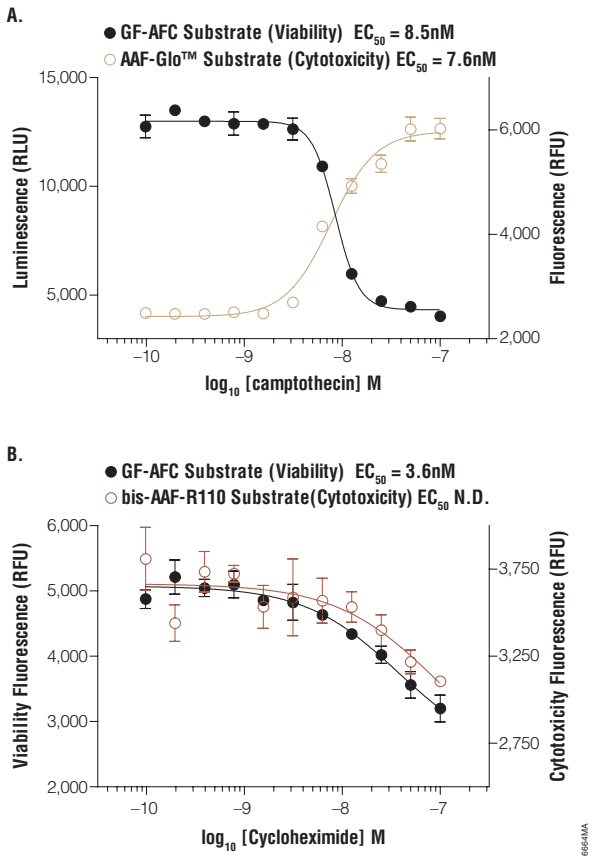



**Figure 7. Complementary $EC_{50}$ values as assay controls.** Jurkat cells were subjected to the indicated concentrations of camptothecin or cycloheximide for 24 hours at 37°C. **Panel A.** MultiTox-Glo Reagent was delivered and resulting signals measured as described previously. The decline in viable cell number was measured using GF-AFC substrate, and increase in cytotoxicity was measured using AAF-Glo™ Substrate. The highest concentrations of camptothecin indicate that camptothecin causes cytotoxicity. **Panel B.** MultiTox-Fluor Reagent was added and fluorescent responses measured. The decline in AFC fluorescence does not correspond to a increase in R110 fluorescence. Visual morphology confirmed that the highest dosages of cycloheximide caused cell-cycle arrest, not cytotoxicity. Therefore the "decrease" in the marker for viable cell number with the most concentrated cycloheximide may reflect arrested cell division compared to the no-treatment control.

effects skewing the apparent potencies (Figure 7). Single-parameter cytotoxicity or viability assays do not have this "built-in" control to help identify problematic data.

## Perform Multiplex Assays for More Information

The CytoTox-Fluor™ and MultiTox-Fluor Cytotoxicity Assay Reagent can be multiplexed with other endpoint assays for additional content on a per well basis. For instance, the MultiTox-Fluor Multiplex Cytotoxicity Assay can be used prior to any luminescence assay (ATP, reporter, or caspase assays). These multiplexes can be of particular value in determining the mechanism of cell death that a compound or treatment produces (Figure 8).

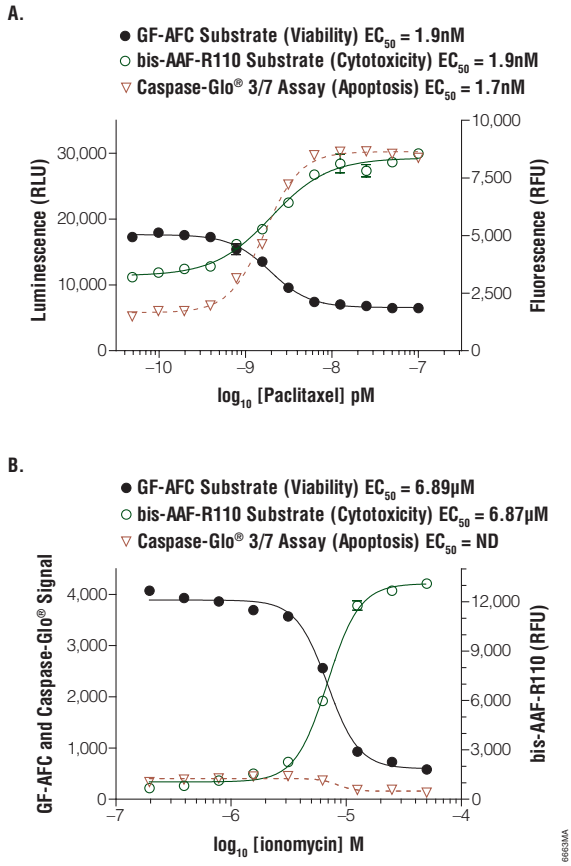



**Figure 8. Additional multiplexes increase informational content.** Jurkat cells were subjected to increasing concentrations of either paclitaxel (24 hours) or ionomycin (6 hours). MultiTox-Fluor Reagent was added and data collected. Caspase-Glo® 3/7 Reagent was added and luminescence measured. **Panel A.** Paclitaxel causes cytotoxicity by apoptosis (caspase induction). **Panel B.** Ionomycin causes cytotoxicity, but does not cause caspase induction (likely to be primary necrosis).

## Summary

The CytoTox-Glo™, MultiTox-Fluor and MultiTox-Glo Multiplex Cytotoxicity Assays represent a significant step forward in viability and cytotoxicity assay technology. The assays provide rapid and sensitive detection of viability and cytotoxicity in nonlytic, homogeneous and scalable formats. The assays offer additional flexibility with either fluorescent and/or luminescent signal readouts. The assays can deliver ratiometric responses that can act as an internal control for cell number normalization or assay interferences. Furthermore, the MultiTox-Fluor and CytoTox-Fluor™ assay systems provide the additional functionality and flexibility to enable multiplexing with other assays.

## References


1. Niles, A. *et al.* (2006) *Cell Notes* **15**, 11–5.
2. Niles, A. *et al.* (2006) *Cell Notes* **16**, 12–5.
3. Niles, A. *et al.* (2006) *Promega Notes* **94**, 22–6.
4. Riss, T.L. and Moravec, R.A. (2004) *ASSAY Drug Dev. Tech.* **2**, 51–62.
5. Zhang, J.H., Chung, T.D., and Oldenberg, R. *et al.* (1999) *J. Biomol. Screen.* **4**, 67–73.


## Protocols

*CytoTox-Fluor™ Cytotoxicity Assay Technical Bulletin* #TB350 (www.promega.com/tbs/tb350/tb350.html)

*MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin* #TB348 (www.promega.com/tbs/tb348/tb348.html)

## Ordering Information

| Product | Size | Cat.# |
|---|---|---|
| MultiTox-Fluor Multiplex Cytotoxicity Assay | 10ml* | G9200 |
| Cytotox-Fluor™ Cytotoxicity Assay | 10ml* | G9260 |
| Cytotox-Glo™ Cytotoxicity Assay | Available Soon | |
| MultiTox-Glo Multiplex Cytotoxicity Assay | Available Soon | |

*Additional Sizes Available

[a]Patent Pending.



MultiTox-Glo and CytoTox-Glo™ Assays will be available soon. Contact Technical Services for more information on these assays. **techserv@promega.com**


**Promega Corporation**
2800 Woods Hollow Road
Madison, WI 53711-5399 *USA*
Tel: 608-274-4330
Fax: 608-277-2516
Toll-Free: 800-356-9526
Toll-Free Fax: 800-356-1970
Internet: www.promega.com

**Promega Biosciences, Inc.**
A Division of Promega Corporation
San Luis Obispo, California

***Australia,* Sydney**
Tel: 02 9565 1100
Fax: 02 9550 4454
Freecall: 1800 225 123
Freefax: 1800 626 017
E-mail: aus_custserv@promega.com

***China,* Beijing**
Tel: (86) 10 5825 6268
Fax: (86) 10 5825 6160
E-mail: promega@promega.com.cn

***France,* Lyon**
Tel: 04 37 22 50 00
Fax: 04 37 22 50 10
Numero Vert: 0 800 48 79 99
E-mail: fr_custserv@fr.promega.com

***Germany/Austria,* Mannheim**
Tel: (+49) (0) 621 8501 0
Fax: (+49) (0) 621 8501 222
Free Phone: 00800 77663422
Free Fax 00800 77663423
E-mail: de_custserv@promega.com

***Italy,* Milan**
Tel: 02 54 05 01 94
Fax: 02 55 18 56 64
Numero Verde: 800 69 18 18
E-mail: it_custserv@it.promega.com

***Japan,* Tokyo**
Tel: 03 3669 7981
Fax: 03 3669 7982
E-mail: jptechserv@jp.promega.com

***Latin America Region,* Brazil**
Tel/Fax: (55 31) 3262 2915
E-mail: latinoam@promega.com

***Belgium/Luxembourg/ The Netherlands,* Leiden**
Tel: (+31) (0) 71 5324244
Fax: (+31) (0) 71 5324907
Free Tel BE: 0800 18098
Free Fax BE: 0800 16971
Free Tel NL: 0800 0221910
Free Fax NL: 0800 0226545
E-mail: bnl_custserv@nl.promega.com

***Pacific Asia Region,* Singapore**
Tel: 65 6513 3450
Fax: 65 6773 5210
E-mail: sg_custserv@promega.com

***Spain,* Madrid**
Tel: 902 538 200
Fax: 902 538 300
E-mail: esp_custserv@promega.com

***Switzerland,* Wallisellen**
Tel: 044 878 90 00
Fax: 044 878 90 10
E-mail: ch_custserv@promega.com

***United Kingdom,* Southampton**
Tel: 023 8076 0225
Fax: 023 8076 7014
Free Phone: 0800 378994
E-mail: ukcustserve@promega.com