Technical Bulletin

# MultiTox-Fluor Multiplex Cytotoxicity Assay

INSTRUCTIONS FOR USE OF PRODUCTS G9200, G9201 AND G9202.

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# MultiTox-Fluor Multiplex Cytotoxicity Assay

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## 1. Description

The MultiTox-Fluor Multiplex Cytotoxicity Assay[a] is a single-reagent-addition fluorescent assay that simultaneously measures the relative number of live and dead cells in cell populations. The MultiTox-Fluor Multiplex Cytotoxicity Assay gives ratiometric, inversely correlated measures of cell viability and cytotoxicity. The ratio of viable cells to dead cells is independent of cell number and, therefore, can be used to normalize data. Having complementary cell viability and cytotoxicity measures reduces errors associated with pipetting and cell clumping. Assays often are subject to chemical interference by test compounds and medium components and can give false-positive or false-negative results. Independent cell viability and cytotoxicity assay chemistries serve as internal controls and allow identification of errors resulting from chemical interference from test compounds or medium components.

The MultiTox-Fluor Assay simultaneously measures two protease activities: one is a marker of cell viability, and the other is a marker of cytotoxicity (Figure 1). The live-cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC). The substrate enters intact cells where it is

## 1. Description (continued)

cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells (Figure 2). 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-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. 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.

The MultiTox-Fluor Assay accommodates downstream multiplexing with many Promega luminescent assays or spectrally distinct fluorescent assays, such as those measuring caspase activation, reporter expression or orthogonal measures of viability.

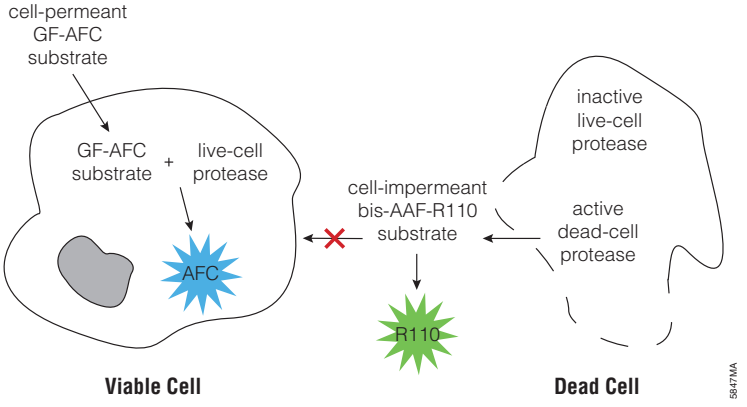


**Figure 1. The biology of the MultiTox-Fluor Multiplex Cytotoxicity Assay.** The GF-AFC Substrate can enter live cells where it is cleaved by the live-cell protease to release AFC. The bis-AAF-R110 Substrate cannot enter live cells but can be cleaved by the dead-cell protease activity to release R110.

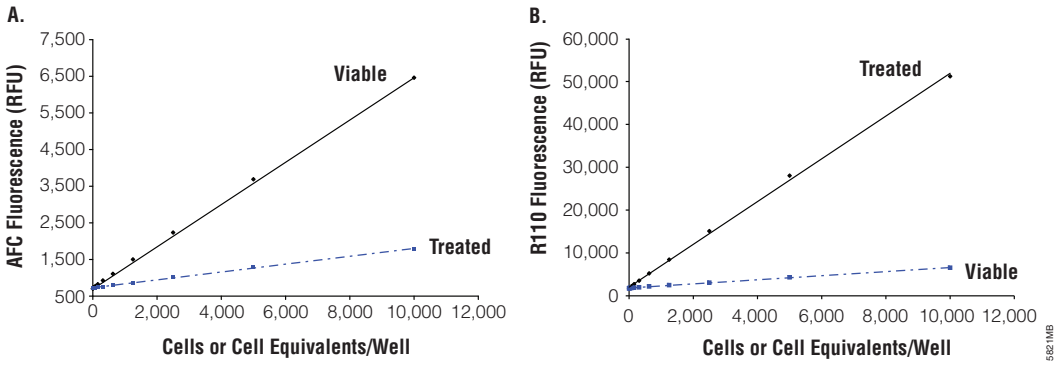


**Figure 2. The MultiTox-Fluor Assay measures distinct and differential protease activities.** Live-cell and dead-cell protease activities were measured in populations of viable cells (untreated) or cells lysed by sonication (treated) using the GF-AFC Substrate (live-cell protease substrate) and bis-AAF-R110 Substrate (dead-cell protease substrate). **Panel A**. GF-AFC fluorescence. **Panel B**. bis-AAF-R110 fluorescence. Note that the Y-axis scales in Panels A and B are different. Results are provided in relative fluorescence units (RFU), and the scales reflect the difference in signal output of AFC and R110.

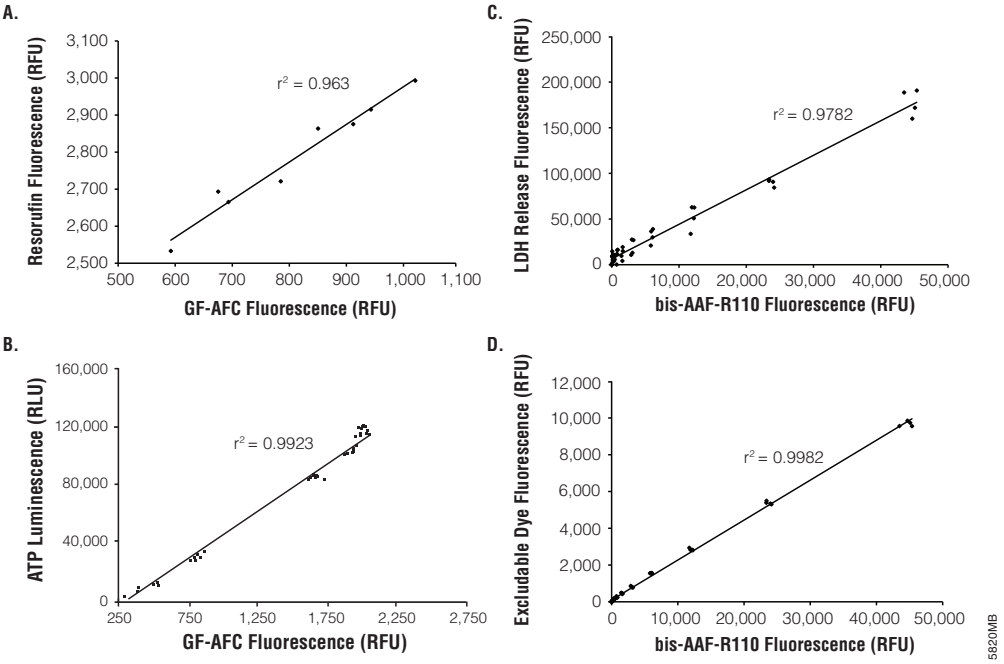


**Figure 3. Live- and dead-cell protease activities detected using the MultiTox-Fluor Assay show strong correlation with established methods for measuring viability and cytotoxicity. Panel A.** bis-AAF-R110 signal plotted against CellTiter-Blue® Assay fluorescence. **Panel B.** GF-AFC signal plotted against CellTiter-Glo® Assay luminescence. **Panel C.** GF-AFC signal plotted against CytoTox-ONE™ Assay fluorescence. **Panel D.** bis-AAF-R110 signal plotted against ethidium homodimer fluorescence.

## 1. Description (continued)

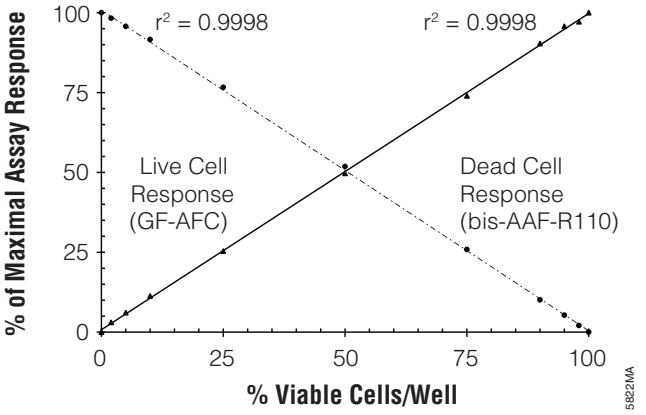


**Figure 4. Viability and cytotoxicity measurements are inversely correlated and ratiometric.** When viability is high, the live-cell signal is highest and the dead-cell signal is lowest. When viability is low, the live-cell signal is lowest and the dead-cell signal is highest. Solid line, live-cell signal; dotted line, dead-cell signal.

### Assay Advantages

- **Simultaneously Measure the Number of Live Cells and Number of Dead Cells in Culture:** Single-reagent-addition, "add-mix-measure" protocol.
- **Normalize Data with a Built-In Internal Control:** The ratio of number of live cells to number of dead cells is independent of cell number and normalizes data. This normalization makes results more comparable well-to-well, plate-to-plate and day-to-day.
- **Identify More False Positives and False Negatives Immediately:** Independent cell viability and cytotoxicity chemistries serve as controls for each other. If test compounds interfere with one assay chemistry, the other serves as an internal control.
- **Get More Data from Every Well:** Multiplex the MultiTox-Fluor Assay with many Promega bioluminescent assays (apoptosis determination, reporter gene and protease activity assays, etc.).

An overview of the protocol is provided in Figure 5.

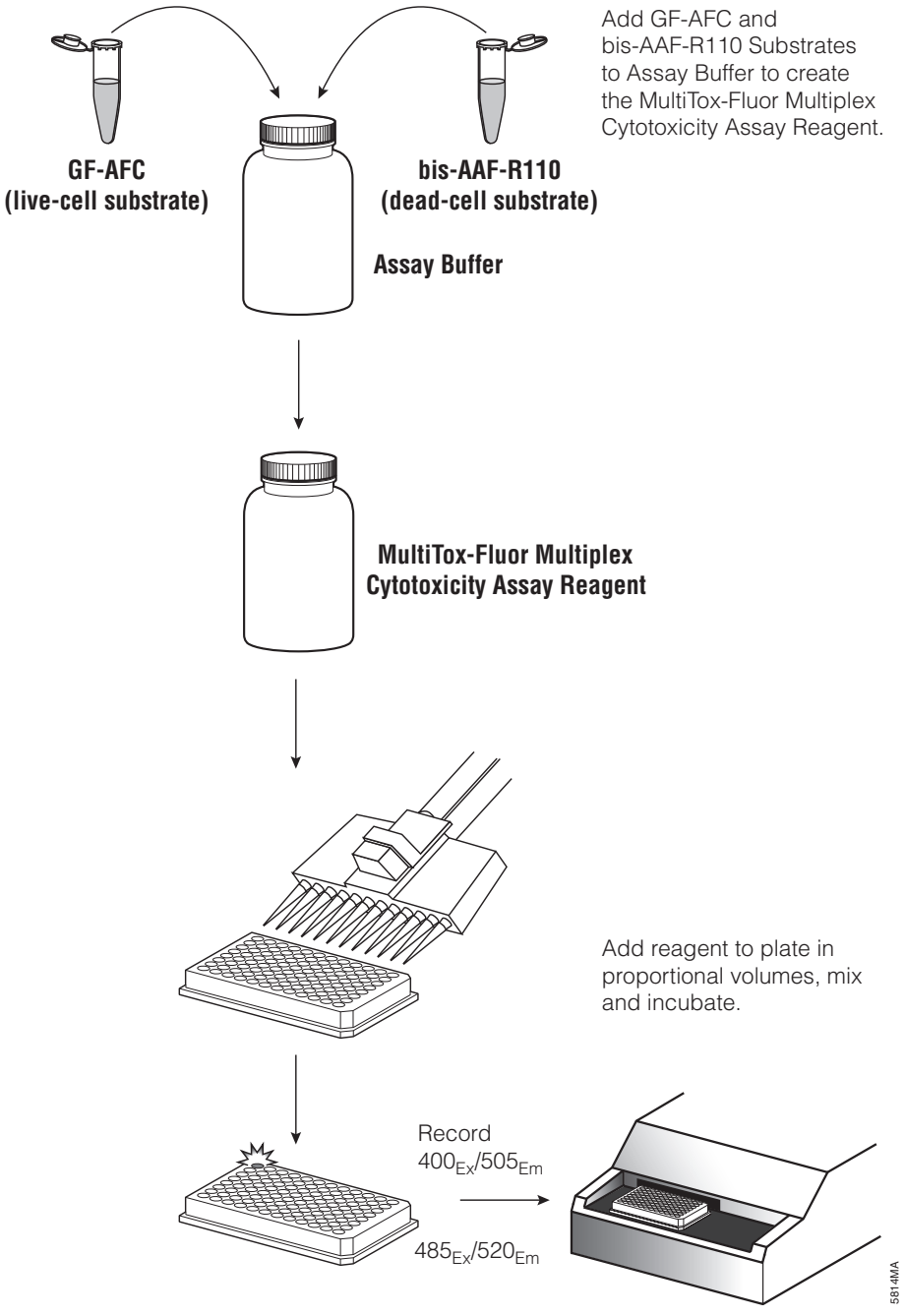


**Figure 5. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay.** Live-cell fluorescence is measured at $400_{Ex}/505_{Em}$; dead-cell fluorescence is measured at $485_{Ex}/520_{Em}$.

## 2. Product Components and Storage Conditions

| Product | Size | Cat.# |
|---|---|---|
| MultiTox-Fluor Multiplex Cytotoxicity Assay | 10ml | G9200 |

G9200 contains sufficient reagents for 100 assays at 100µl/assay in a 96-well format or 400 assays at 25µl/assay in a 384-well format. Includes:

- 1 × 10ml Assay Buffer (Part# G610A)
- 1 × 10µl GF-AFC Substrate
- 1 × 10µl bis-AAF-R110 Substrate

| Product | Size | Cat.# |
|---|---|---|
| MultiTox-Fluor Multiplex Cytotoxicity Assay | 5 × 10ml | G9201 |

G9201 contains sufficient reagents for 500 assays at 100µl/assay in a 96-well format or 2,000 assays at 25µl/well in a 384-well format. Includes:

- 5 × 10ml Assay Buffer (Part# G610A)
- 5 × 10µl GF-AFC Substrate
- 5 × 10µl bis-AAF-R110 Substrate

| Product | Size | Cat.# |
|---|---|---|
| MultiTox-Fluor Multiplex Cytotoxicity Assay | 2 × 50ml | G9202 |

G9202 contains sufficient reagents for 1,000 assays at 100µl/assay in a 96-well format or 4,000 assays at 25µl/well in a 384-well format. Includes:

- 2 × 50ml Assay Buffer (Part# G610B)
- 2 × 50µl GF-AFC Substrate
- 2 × 50µl bis-AAF-R110 Substrate

**Storage Conditions:** Store the MultiTox-Fluor Multiplex Cytotoxicity Assay components at –20°C. See product label for expiration date information.

**Note:** The GF-AFC Substrate and bis-AAF-R110 Substrate are supplied at 100mM in DMSO.

## 3. Reagent Preparation and Storage

The MultiTox-Fluor Reagent can be prepared and used at two different concentrations, depending on the intended application. The 2X concentration is preferred for standard viability and cytotoxicity determinations. The 5X concentration is required to accommodate volume restraints when multiplexing with other compatible downstream assay chemistries.

1. Thaw the MultiTox-Fluor Multiplex Cytotoxicity Assay components in a 37°C water bath. The substrate vials may require a brief centrifugation to recover the entire volume.

2a. To make the 2X reagent, transfer the contents of the GF-AFC and bis-AAF-R110 Substrates (10µl each for Cat.# G9200 and G9201; 50µl for G9202) to the Assay Buffer (10ml for Cat.# G9200 and G9201; 50ml for G9202). Mix by vortexing the contents until the substrates are thoroughly dissolved. The 2X reagent should be used in equal-volume additions (i.e., 100µl for 96-well plates; 25µl for 384-well plates).

OR

2b. To make the 5X reagent, transfer the contents of the GF-AFC and bis-AAF-R110 Substrates to 2.0ml of Assay Buffer (96-well plates) or 2.5ml of Assay Buffer (384-well plates) for Cat.# G9200 and G9201. [Transfer the substrates to 10ml of Assay Buffer for Cat.# G9202.] Mix by vortexing until the substrates are thoroughly dissolved. The 5X reagent should be used in 1/5 volume additions, (i.e., 20µl for 96-well plates and 5µl for 384-well plates).

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

## 4. Protocols

**Materials to Be Supplied by the User**

- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reagent reservoirs
- fluorescence plate reader equipped with filter sets of excitation ~400nm and emission ~505nm, and excitation ~485nm and emission ~520nm
- orbital plate shaker
- positive control cytotoxic reagent or lytic detergent (e.g., digitonin at 20mg/ml in DMSO)

If you have not performed this assay on your cell line previously, we strongly recommend determining assay sensitivity using your cells and one of the two methods described in Section 4.D or 4.E. A description of recommended controls can be found in Section 4.C.

### 4.A. Example Cytotoxicity and Viability Assay Protocol

1. Set up 96-well assay plates containing cells in culture medium at the desired density.
2. Add test compounds and vehicle controls to the appropriate wells so that the final volume is 100µl in each well (25µl for a 384-well plate).
3. Culture cells for the desired test exposure period.
4. Add the 2X MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent in an equal volume (100µl per well) to all wells. Mix briefly on an orbital shaker, then incubate for at least 30 minutes at 37°C.

   **Note:** Longer incubations may improve assay sensitivity and dynamic range. **Do not** incubate longer than 3 hours.
5. Measure fluorescence:
   - Viability: Excitation ~400nm; Emission ~505nm
   - Cytotoxicity: Excitation ~485nm; Emission ~520nm

   **Notes:**

   Adjusting the instrument gain setting (applied photomultiplier tube energy) may be necessary.

   Data are collected as relative fluorescence units (RFU), and the scales for AFC and R110 fluorescence will differ due to the difference in signal output of the two molecules (Figure 2).

### 4.B. Example Multiplex Protocol (with a luminescent caspase assay)

Representative data are shown in Figure 6.

1. Set up 96-well assay plates containing cells in medium at the desired density.
2. Add test compounds and vehicle controls to the appropriate wells so that the final volume in each well is 100µl (25µl for a 384-well plate).
3. Culture cells for the desired test exposure period.
4. Add 20µl of 5X MultiTox-Fluor Reagent **(prepared as 10µl of each substrate in 2ml of Assay Buffer)** to each well, and mix briefly by orbital shaking. Incubate for at least 30 minutes at 37°C.

   **Note:** Longer incubations may improve assay sensitivity and dynamic range. **Do not** incubate longer than 3 hours.
5. Measure fluorescence using a fluorometer (live-cell fluorescence at $400_{Ex}/505_{Em}$; dead-cell fluorescence at $485_{Ex}/520_{Em}$).
6. Add an equal volume of Caspase-Glo® 3/7 Reagent to each well (100–120µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

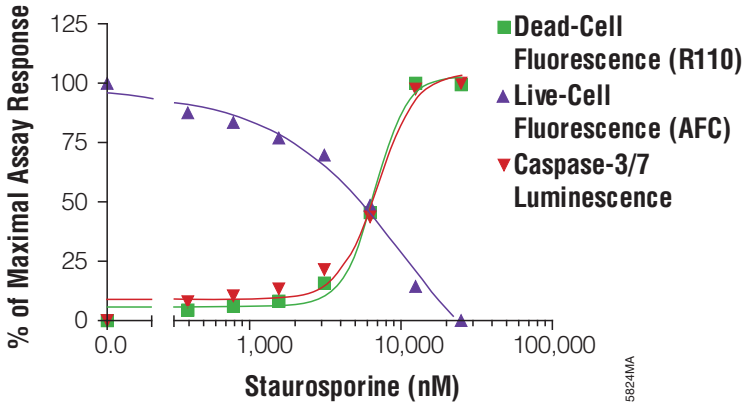


**Figure 6. The MultiTox-Fluor Assay can be multiplexed with other assays.** LN-18 cells were plated at a density of 10,000 cells per well in 50µl volumes of MEM + 10% fetal bovine serum and allowed to attach overnight. Staurosporine was serially diluted twofold and added to wells in 50µl volumes. The plate was incubated at 37°C in 5% $CO_2$ for 6 hours. MultiTox-Fluor Reagent was prepared by combining 10µl of each substrate with 1ml of Assay Buffer, and 10µl was used per well. The plate was mixed and incubated for 30 minutes at 37°C. Fluorescence was measured at $400_{Ex}/505_{Em}$ and $485_{Ex}/520_{Em}$ using a BMG PolarStar plate reader. Caspase-Glo® 3/7 Reagent then was added in an additional 100µl volume, and luminescence was measured after a 10-minute incubation. The resulting signals were normalized to a percentage of maximal response and plotted using GraphPad Prism® software.

### 4.C. Recommended Controls

**No-Cell Control:** Set up triplicate wells without cells to serve as the negative control to determine background fluorescence.

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

**Test Compound Control (optional):** Set up triplicate wells without cells, and add the vehicle and test compound to test for possible fluorescence interference.

**Positive Control for 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., treat cells with digitonin at a final concentration of 30µg/ml for 15 minutes).

### 4.D. Determining Assay Sensitivity, Method 1

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium (to remove residual trypsin) and resuspend in fresh medium.

   **Note:** For cells growing in suspension, proceed directly to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, then adjust the concentration by dilution to 100,000 viable cells/ml in at least 3.0ml of fresh medium.

   **Note:** Concentration by centrifugation may be necessary if the cell suspension is less dense than 100,000 cells/ml.

3. Add 100µl of the 100,000 cell/ml dilution (10,000 cells/well) into each well of rows A and B of a 96-well plate. See Table 1.

**Table 1. Schematic Diagram of 96-Well Plate Layout.**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 10,000 Cells/Well | | | | | | | | | | | |
| **B** | 5,000 Cells/Well | | | | | | | | | | | |
| **C** | 2,500 Cells/Well | | | | | | | | | | | |
| **D** | 1,250 Cells/Well | | | | | | | | | | | |
| **E** | 625 Cells/Well | | | | | | | | | | | |
| **F** | 313 Cells/Well | | | | | | | | | | | |
| **G** | 156 Cells/Well | | | | | | | | | | | |
| **H** | 0 Cells/Well | | | | | | | | | | | |
| | untreated | | | | | | treated | | | | | |

4. Add 100µl of fresh medium to each well in rows B–H.

5. Using a multichannel pipettor, mix the cell suspensions in row B by pipetting. Be careful not to create foam or bubbles. Transfer a 100µl volume from row B to row C. Repeat mixing, and transfer 100µl from row C to row D. Continue this process to row G. After mixing the cell suspension in row G, remove 100µl from the wells and discard it. This procedure dilutes the cells from 10,000 cells/well in row A to 156 cells/well in row G. Wells in row H serve as no-cell background controls.

6. Dilute digitonin to 300µg/ml in water. Using a multichannel pipette, carefully add 10µl of the digitonin solution to all wells in columns 7–12 to lyse cells (treated cells). Add 10µl of water to all wells in columns 1–6 to normalize the volume in all wells (untreated cells).

7. Add 100µl of the MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent to each well, mix briefly by orbital shaking to ensure homogeneity and incubate at 37°C for at least 30 minutes. Protect plates from light.

   **Note:** Longer incubations may improve assay sensitivity and dynamic range. **Do not** incubate longer than 3 hours.

8. Measure fluorescence:

   • Viability (live-cell fluorescence): Excitation ~400nm; Emission ~505nm
   • Cytotoxicity (dead-cell fluorescence): Excitation ~485nm; Emission ~520nm

   **Note:** Adjusting the instrument gain setting (applied photomultiplier tube energy) may be necessary.

9. Calculate the signal-to-noise ratios (S:N) to determine practical sensitivity for your cell type for each dilution of cells (10,000 cells/well, 5,000 cells/well, 2,500 cells/well, etc.)

   $$\text{Viability S:N} = \frac{\text{(Average Untreated, RFU – Average Treated, RFU)}}{\text{S.D. of H-1 through H-12}}$$

   $$\text{Cytotoxicity S:N} = \frac{\text{(Average Treated, RFU – Average Untreated, RFU)}}{\text{S.D. of H-1 through H-12}}$$

   **Note:** The practical level of assay sensitivity for either assay is a signal-to-noise ratio of greater than 3 standard deviations (derived from reference 1).

### 4.E. Determining Assay Sensitivity, Method 2

1. Harvest adherent cells (by trypsinization, etc.), wash with fresh medium (to remove residual trypsin) and resuspend in fresh medium.

   **Note:** For cells growing in suspension, proceed directly to Step 2.

2. Determine the number of viable cells by trypan blue exclusion using a hemacytometer, then adjust the concentration by dilution to 100,000 viable cells/ml in at least 20ml of fresh medium.

   **Note:** Concentration by centrifugation may be necessary if the cell suspension is less dense than 100,000 cells/ml.

3. Divide the volume of cells into two separate tubes. Subject one tube to "moderate" sonication (empirically determined by post-sonication morphological examination) to disrupt cell membrane integrity and simulate a 100% cytotoxic population. The second tube of untreated cells will serve as the maximum viable population.

**4.E. Determining Assay Sensitivity, Method 2 (continued)**

4. Create a spectrum of viability by blending sonicated and untreated cell populations in 1.5ml microcentrifuge tubes as described in Table 2.

**Table 2. Spectrum of Viability Generated by Blending Sonicated and Untreated Cells.**

| Percent Viability | Volume of Sonicated Cells (µl) | Volume of Untreated Cells (µl) |
|---|---|---|
| 100 | 0 | 1,000 |
| 95 | 50 | 950 |
| 90 | 100 | 900 |
| 75 | 250 | 750 |
| 50 | 500 | 500 |
| 25 | 750 | 250 |
| 10 | 900 | 100 |
| 5 | 950 | 50 |
| 0 | 1,000 | 0 |

5. After mixing each blend by gently vortexing, pipet 100µl of each blend into eight replicate wells of a 96-well plate. Add the 100% viable sample to column 1, 95% viable to column 2, etc. Add cell culture medium only to column 10 to serve as the no-cell control.

6. Add MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent in an equal volume (100µl per well) to all wells, mix briefly by orbital shaking and incubate for at least 30 minutes at 37°C

   **Note:** Longer incubations may improve assay sensitivity and dynamic range. **Do not** incubate longer than 3 hours.

7. Measure fluorescence:

   - Viability (live-cell fluorescence): Excitation ~400nm; Emission ~505nm
   - Cytotoxicity (dead-cell fluorescence): Excitation ~485nm; Emission ~520nm

   **Note:** Adjusting the instrument gain setting (applied photomultiplier tube energy) may be necessary.

8. Determine the practical sensitivity for your cell type by calculating the signal-to-noise ratio (S:N) for each blend of cell viability (X = 95, 90% etc.)

$$\text{Viability S:N} = \frac{(\text{Average X\%, RFU} - \text{Average 0\%, RFU})}{\text{S.D. of 0\%}}$$

$$\text{Cytotoxicity S:N} = \frac{(\text{Average X\%, RFU} - \text{Average 100\%, RFU})}{\text{S.D. of 100\%}}$$

**Note:** The practical level of assay sensitivity for either assay is a signal-to-noise ratio of greater than 3 standard deviations (derived from reference 1).

## 5. General Considerations

### Background Fluorescence and Inherent Serum Activity

Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used for dead-cell measurements. The level of this protease activity may vary among different lots of serum. To correct for this variability, background fluorescence should be determined using samples containing medium plus serum without cells.

### Temperature

Generation of the fluorescent product is proportional to the protease activity of the markers associated with cell viability and cytotoxicity. The activities of these proteases are influenced by temperature. For best results, we recommend incubating assays at a constant controlled temperature to ensure uniformity across the plate. After reagent addition and a brief mixing, we suggest one of two methods:

1. Incubate at 37°C in a water-jacketed incubation module (Me'Cour, etc.).

   **Note:** Incubation at 37°C in a $CO_2$ culture cabinet may lead to edge effects due to thermal gradients.

2. Incubate at room temperature with or without orbital shaking.

   **Note:** Assays performed at room temperature may require more than 30 minutes of incubation. **Do not** incubate longer than 3 hours.

### Assay Controls

In addition to a no-cell control to establish background fluorescence, we recommend including an untreated-cell (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by adding vehicle only. Vehicle is used to deliver the test compound to test wells and, in most cases, consists of a buffer system or medium and the equivalent amount of solvent added with the test compound. Maximum cytotoxicity can be determined using a compound that causes cytotoxicity or a lytic reagent added to compromise viability (e.g., nonionic detergents such as digitonin or zwitterionic detergents). See Section 4.C.

Triton® X-100, NP-40 and SDS interfere with the assay.

### Cytotoxicity Marker Half-Life

The activity of the protease marker released from dead cells has a half-life of approximately 9–10 hours (2). In situations where cytotoxicity occurs very rapidly (necrosis) and the treatment time is greater than 24 hours, the degree of cytotoxicity may be underestimated. Addition of a lytic detergent may be useful to determine the total cytotoxicity marker activity remaining (from remaining live cells) in these extended treatments.

## 5. General Considerations (continued)

### Light Sensitivity

The MultiTox-Fluor Multiplex Cytotoxicity Assay uses two fluorogenic peptide substrates. Although the substrates demonstrate good general photostability, the fluors liberated after contact with the proteases can degrade with prolonged exposure to ambient light sources. We recommend shielding the plates from ambient light at all times.

### Cell Culture Medium

The GF-AFC and bis-AAF-R110 Substrates are introduced into the test well using an optimized buffer system that mitigates differences in pH due to the treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of medium formulations with very high serum content or phenol red indicator levels, no substantial performance differences will be observed among media.

### Instrument Settings and Measurement Parameters

To acquire both cell viability and cytotoxicity readings from one Multitox-Fluor reaction, the instrument must have the recommended filter sets. An incorrect filter set will result in inadequate signal separation or high background readings. If the recommended filter sets are not readily available, contact Promega Technical Services for alternatives.

Many fluorescent microplate readers have a detection gain setting. Setting the detection gain too low can result in low signal and output with high variation (i.e., large error bars). Setting the detection gain too high may result in signal plateau (i.e., signal saturation) and inadequate signal separation. See Section 6.

## 6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

| Symptoms | Causes and Comments |
|---|---|
| Little difference in fluorescence between treated and untreated cell populations (Figure 7) | Improper filter set. Measure live-cell fluorescence at $400_{Ex}/505_{Em}$ and dead-cell fluorescence at $485_{Ex}/520_{Em}$. |
| | Treatment did not induce cytotoxicity. Check the positive control for cytotoxicity; there should be a significant difference in signal between the treated and untreated cell populations. |

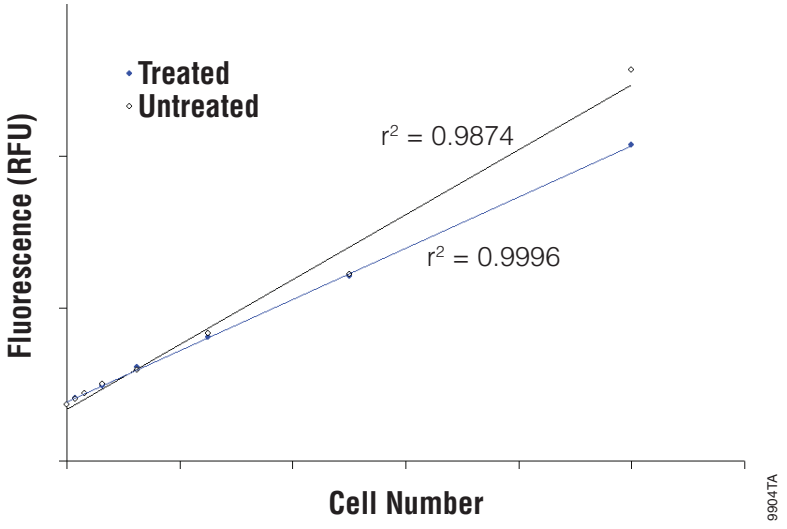


**Figure 7. An example showing little difference in GF-AFC fluorescence between treated (dead) and untreated (viable) cell populations.** Fluorescence increases with increasing cell number, but there is little difference in fluorescence between treated and untreated cell populations.

## 6. Troubleshooting (continued)

| Symptoms | Causes and Comments |
|---|---|
| Little change in fluorescence with increasing cell number for both treated and untreated cell populations (Figure 8) | Improper filter set. Measure live-cell fluorescence at $400_{Ex}/505_{Em}$ and dead-cell fluorescence at $485_{Ex}/520_{Em}$. |

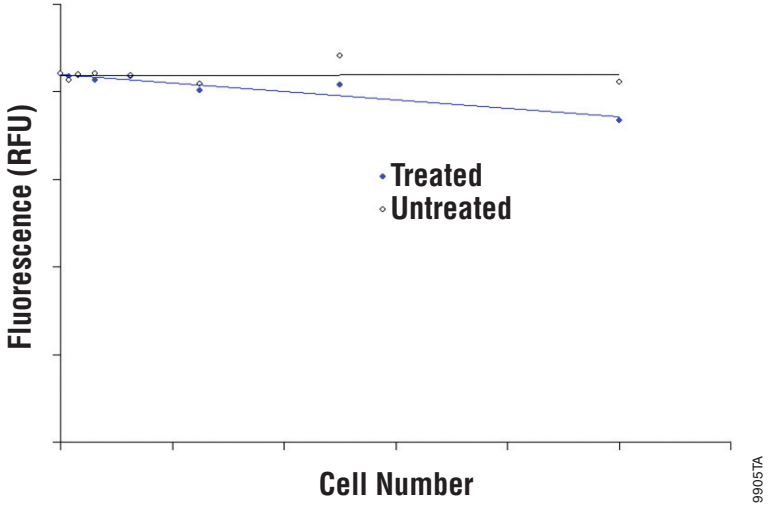


**Figure 8. An example showing little change in GF-AFC fluorescence with increasing cell number for both treated and untreated cell populations.** Fluorescence is relatively high for both treated and untreated cells at low cell numbers and does not change dramatically with increasing cell number.

| Symptoms | Causes and Comments |
|---|---|
| High variation (i.e., large error bars) and low fluorescence despite increasing cell number (Figure 9) | The instrument gain setting was too low. Increase the gain setting. |
| | Mixing of reagent and cells was inadequate. Mix reagent and cells gently on an orbital platform. |

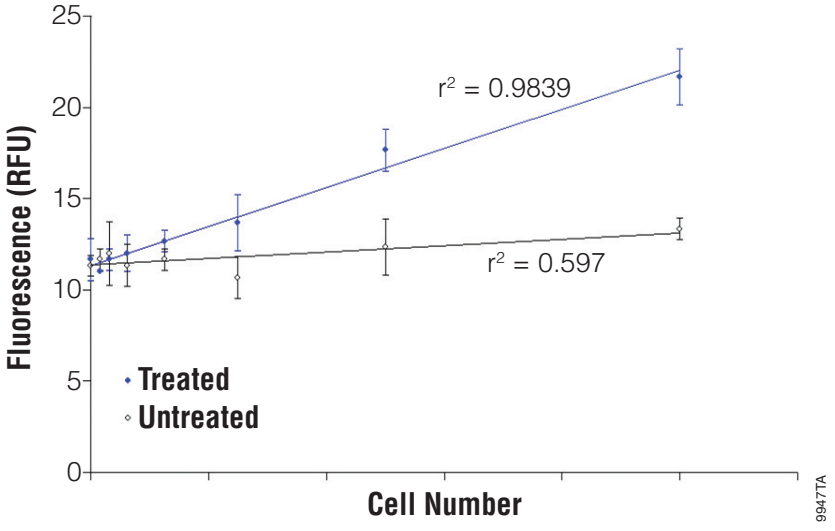


**Figure 9. An example showing high variation and low bis-AAF-R110 fluorescence despite increasing cell number.**

## 6. Troubleshooting (continued)

| Symptoms | Causes and Comments |
|---|---|
| Fluorescence reached plateau for one or both cell populations (Figure 10) | The instrument gain setting was too high, and the signal reached saturation. Reduce the gain setting. |
| | Fluorescence was too high, and signal reached saturation. Be sure fluorescence levels are within the linear range of the detection instrument. |
| Narrow signal window (i.e., small difference in signal between treated and untreated cell populations) (Figure 11) | Incubation time was inadequate. Incubate reactions for at least 30 minutes at 37°C. An additional incubation time of up to 3 hours often improves results. **Do not** incubate longer than 3 hours. |
| | Improper filter set. Measure live-cell fluorescence at $400_{Ex}/505_{Em}$ and dead-cell fluorescence at $485_{Ex}/520_{Em}$. |
| | Improper instrument gain setting. |
| Live-cell signal decreased, but dead-cell signal also decreased | Improper assay time. The dead cell protease has a half life of approximately 9–10 hours. With prolonged treatment times, the dead-cell protease signal can decay before fluorescence is measured. Optimize the assay time. Measure live-cell and dead-cell protease activities at earlier time points. |
| Live signal decreased, but dead signal remained the same | Cell cycle arrest without secondary necrosis. Extend the treatment to promote cell death and loss of membrane integrity and increase dead-cell protease activity. |
| High variation (i.e., large error bars) in signal between replicate reactions | Mixing of reagent and cells was inadequate. Mix reagent and cells gently on an orbital platform prior to incubation at 37°C. |
| | There was a bubble in the well. Pop any bubbles with a sharp object before measuring fluorescence. |
| $EC_{50}$ values determined using this assay differ from $EC_{50}$ values in the literature | The susceptibility of cells to a drug is affected by many factors such as cell type, treatment time, seeding density and cell passage number. These factors can cause $EC_{50}$ values to be different. |

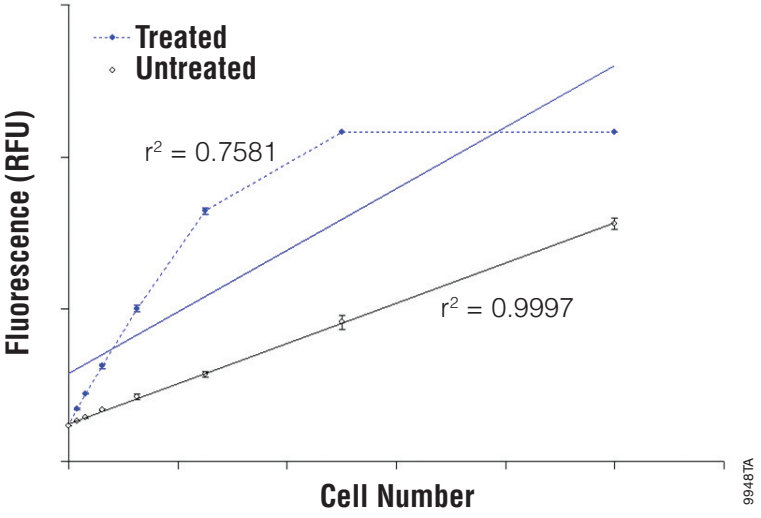


**Figure 10. An example showing signal plateau for treated cells.**

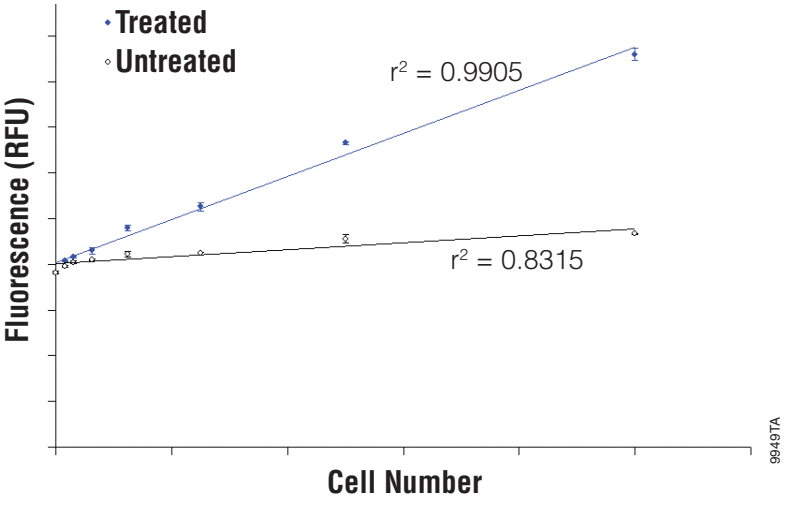


**Figure 11. An example showing a narrow signal window between treated and untreated cell populations.** bis-AAF-R110 fluorescence levels are similar for both treated and untreated cell populations.

## 7. References

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

2. Niles, A.L. *et al.* (2007) A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. *Anal. Biochem.* **366**, 197–206.

## 8. Related Products

### Cell Viability Assays

| Product | Size | Cat.# |
|---|---|---|
| ApoLive-Glo™ Multiplex Assay | 10ml | G6410 |
| ApoTox-Glo™ Triplex Assay | 10ml | G6320 |
| CellTiter-Fluor™ Cell Viability Assay | 10ml | G6080 |
| | 5 × 10ml | G6081 |
| | 2 × 50ml | G6082 |
| CytoTox-Fluor™ Cytotoxicity Assay | 10ml | G9260 |
| CellTiter-Glo® Luminescent Cell Viability Assay | 10ml | G7570 |
| CytoTox-ONE™ Homogeneous Membrane Integrity Assay | 1,000–4,000 assays | G7891 |
| CellTiter-Blue® Cell Viability Assay | 20ml | G8080 |

Additional Sizes Available.

### Apoptosis Assays

| Product | Size | Cat.# |
|---|---|---|
| Caspase-Glo® 3/7 Assay | 100ml | G8092 |
| | 10 × 10ml | G8093 |
| Caspase-Glo® 8 Assay | 100ml | G8202 |
| Caspase-Glo® 9 Assay | 100ml | G8212 |

Additional Sizes Available.

### Reporter Gene Assays

| Product | Size | Cat.# |
|---|---|---|
| ONE-Glo™ Luciferase Assay System | 10ml | E6110 |
| Bright-Glo™ Luciferase Assay System | 10ml | E2610 |
| Steady-Glo® Luciferase Assay System | 10ml | E2510 |

Additional Sizes Available.

### Protease Assays

| Product | Size | Cat.# |
|---|---|---|
| Calpain-Glo™ Protease Assay | 10ml | G8501 |
| DPPIV-Glo™ Protease Assay | 10ml | G8350 |
| Proteasome-Glo™ 3-Substrate Cell-Based Assay System | 10ml | G1180 |

Additional Sizes Available.

### Fluorometers

| Product | Size | Cat.# |
|---|---|---|
| GloMax®-Multi Base Instrument | 1 each | E7031 |
| GloMax®-Multi Fluorescence Module | 1 each | E7051 |
| GloMax®-Multi Optical Kit Blue | 1 each | E8921 |

[a]Patent Pending.





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