Technical Bulletin

CellTiter-Fluor™ Cell Viability Assay

INSTRUCTIONS FOR USE OF PRODUCTS G6080, G6081 AND G6082.

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1. Description

The CellTiter-Fluor™ Cell Viability Assay[a] is a nonlytic, single-reagent-addition fluorescence assay that measures the relative number of live cells in a culture population after experimental manipulation (Figures 1 and 2). The CellTiter-Fluor™ Cell Viability Assay measures a conserved and constitutive protease activity within live cells and therefore serves as a marker of cell viability (1). Results obtained using the CellTiter-Fluor™ Cell Viability Assay correlate well with other established methods of determining cell viability (Figure 3). 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 cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells (Figure 4). This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium.

The CellTiter-Fluor™ Cell Viability Assay also can be used in a single-well, sequential, multiplex format with other downstream chemistries to normalize data by cell number. Data from the assay can serve as an internal control and

allow identification of errors resulting from cell clumping or compound cytotoxicity. The CellTiter-Fluor™ Cell Viability Assay is compatible with most Promega luminescence assays or spectrally distinct fluorescence assay methods, such as assays measuring caspase activation, reporter gene expression or orthogonal measures of viability. However, some P450-Glo™ multiplex protocols may require removing culture supernatant to a separate assay well before performing the assay because of isoform-specific competitive inhibition of the cytotochrome P450 enzymes by the coumarin product of the CellTiter-Fluor™ Cell Viability Assay reaction.

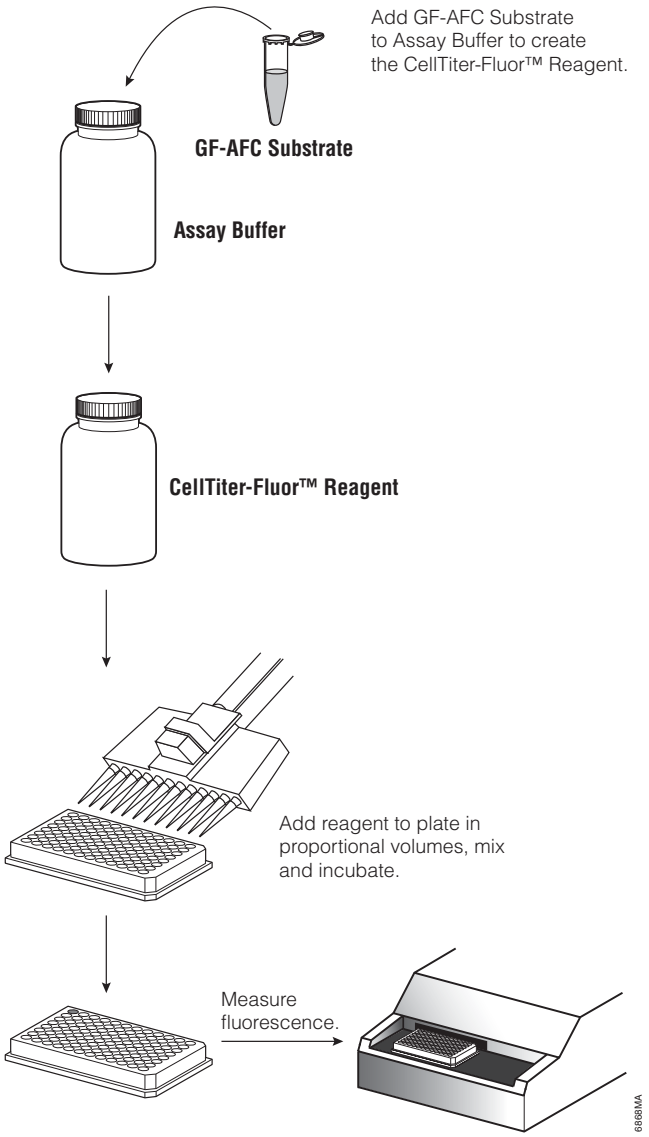

Figure 1. Schematic diagram of the CellTiter-Fluor™ Cell Viability Assay.

Assay Benefits

Measure the Relative Number of Live Cells in Culture: Nonlytic, single-reagent-addition, homogeneous, "add-mix-measure" protocol.

Get More Data from Every Well: The CellTiter-Fluor™ Cell Viability Assay can be performed in multiplex with most Promega luminescence assays.

Normalize Data for Cell Number: Normalizing data for live-cell number makes results more comparable well-to-well, plate-to-plate, day-to-day.

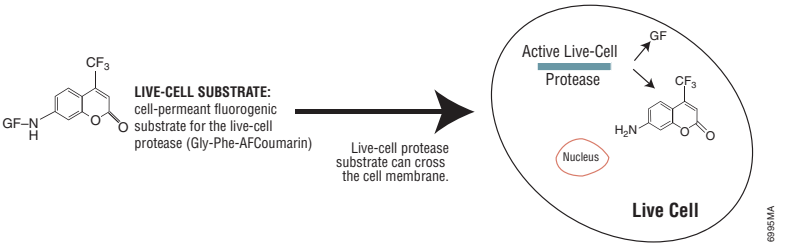

Figure 2. CellTiter-Fluor™ Cell Viability Assay chemistry. The cell-permeant substrate enters the cell, where it is cleaved by the live-cell protease activity to produce the fluorescent AFC. The live-cell protease is labile in membrane-compromised cells and cannot cleave the substrate.

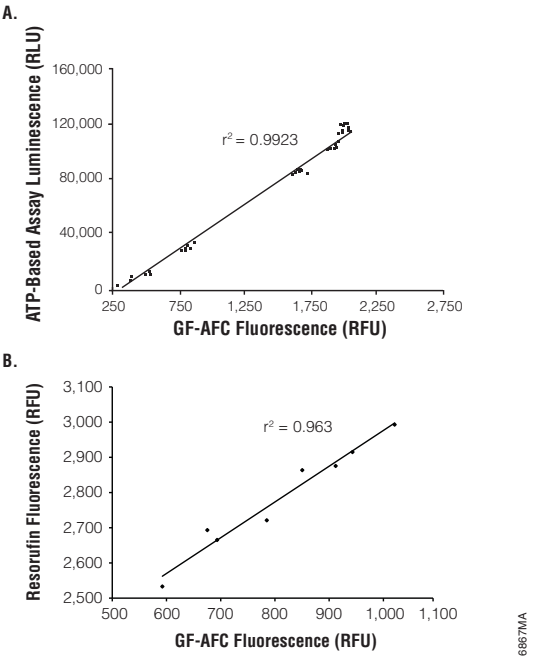

Figure 3. The CellTiter-Fluor™ Cell Viability Assay shows strong correlation with established methods for measuring viability. Panel A. The GF-AFC Substrate signal from serial dilutions of live cells plotted against results from the CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570), which measures cellular ATP. **Panel B.** The GF-AFC Substrate signal from serial dilutions of live cells plotted against results achieved using the CellTiter-Blue® Cell Viability Assay (Cat.# G8080).

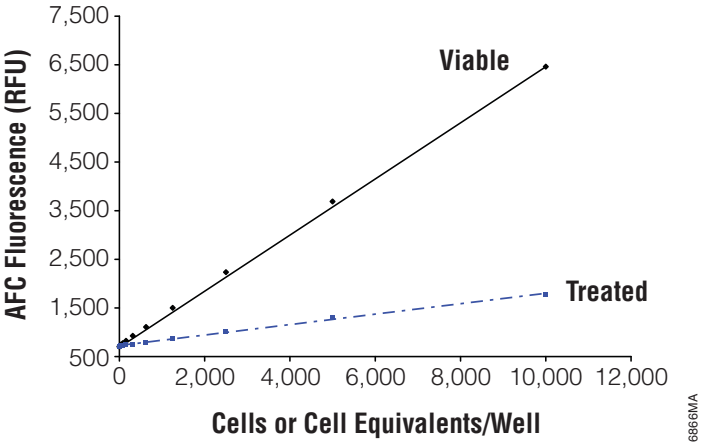

Figure 4. The CellTiter-Fluor™ Cell Viability Assay signal derived from viable cells (untreated) is proportional to cell number. Dead cells (treated) do not contribute appreciable signal in the assay.

2. Product Components and Storage Conditions

Product	Size	Cat.#
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080

Cat.# G6080 contains sufficient reagents for 100 assays at 100µl/assay in a 96-well plate format or 400 assays at 25µl/assay in a 384-well plate format. Includes:

- 1 × 10ml Assay Buffer
- 1 × 10µl GF-AFC Substrate (100mM in DMSO)

Product	Size	Cat.#
CellTiter-Fluor™ Cell Viability Assay	5 × 10ml	G6081

Cat.# G6081 contains sufficient reagents for 500 assays at 100µl/assay in a 96-well plate format or 2,000 assays at 25µl/well in a 384-well format. Includes:

- 5 × 10ml Assay Buffer
- 5 × 10µl GF-AFC Substrate (100mM in DMSO)

Product	Size	Cat.#
CellTiter-Fluor™ Viability Assay	2 × 50ml	G6082

Cat.# G6082 contains sufficient reagents for 1,000 assays at 100µl/assay in a 96-well plate format or 4,000 assays at 25µl/well in a 384-well format. Includes:

- 2 × 50ml Assay Buffer
- 2 × 50µl GF-AFC Substrate (100mM in DMSO)

Storage Conditions: Store the CellTiter-Fluor™ Cell Viability Assay components at –20°C. See product label for expiration date.

3. Reagent Preparation and Storage

1. Completely thaw the CellTiter-Fluor™ Cell Viability Assay components in a 37°C water bath. Vortex the GF-AFC substrate to ensure homogeneity, then briefly centrifuge for complete substrate volume recovery.

2. Transfer the GF-AFC Substrate (10µl for Cat.# G6080 and G6081; 50µl for Cat.# G6082) into the Assay Buffer container (10ml for Cat.# G6080 and G6081; 50ml for Cat.# G6082) to form a 2X Reagent. Mix by vortexing the contents until the substrate is thoroughly dissolved.

   Note: The solution may initially appear "milky" when the GF-AFC substrate is delivered to the buffer. This is normal. The substrate will dissolve with vortexing. The CellTiter-Fluor™ Reagent may be scaled to accommodate the volumes required for downstream multiplexes. To do this, use 1/5 the volume of buffer when you prepare the reagent (i.e., 10µl of the GF-AFC Substrate in 2ml of Assay Buffer). Be sure to label the bottle to indicate that this is a more concentrated reagent, suitable for multiplex assays. Add the reagent at 1/5 the volume of the cell culture.

   Storage: The CellTiter-Fluor™ Viability Reagent should be used within 24 hours if stored at room temperature. Unused GF-AFC Substrate and Assay Buffer can be stored at 4°C for up to 7 days with no appreciable loss of activity.

4. Protocols for the CellTiter-Fluor™ Cell Viability Assay

Materials to Be Supplied by the User

- 96-, 384-, or 1536-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor or liquid-dispensing robot
- reagent reservoirs
- fluorescence plate reader with filter sets for AFC (380–400nm$_{Ex}$/505$_{Em}$)
- orbital plate shaker
- compound known to cause 100% cytotoxicity or lytic detergent (digitonin, Calbiochem Cat.# 300410 or Sigma-Aldrich Cat.# D141 at 20mg/ml in DMSO).

If you have not performed this assay on your cell line previously, we recommend determining assay sensitivity using your cells and one of the two methods described below (Section 4.A or 4.B). If you do not need to determine assay sensitivity for your cells, proceed to Section 4.C.

4.A. 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 to Step 2.

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

   Note: Concentrate the cells by centrifuging and removing medium if the cell suspension is less than 100,000 cells/ml.

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

4. Add 100µl of fresh medium to all wells in rows B–H.

5. Using a multichannel pipettor, mix the cell suspension in row B by pipetting (being careful not to create foaming or bubbles). Transfer 100µl 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 diluted suspension at row G, aspirate 100µl from wells and discard it. This procedure dilutes your cells from 10,000 cell/well in row A to 156 cells/well in row G. Row H will serve as the no-cell, background control.

Table 1. Schematic 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											

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

7. Add 100µl of the CellTiter-Fluor™ Reagent to all wells, mix briefly by orbital shaking and incubate at 37°C for at least 30 minutes.

   Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours, and be sure to shield plates from ambient light.

8. Measure resulting fluorescence with a fluorometer (380–400nm$_{Ex}$/505nm$_{Em}$)

   Note: You may need to adjust instrument gains (applied photomultiplier tube energy).

9. Calculate the practical sensitivity for your cell type by making a signal-to-noise calculation for each dilution of cells (10,000 cells/well; 5,000 cells/well; 2,500 cells/well, etc.).

   $$\text{Viability S:N} = \frac{(\text{Average Untreated} - \text{Average Treated})}{\text{Std. Dev. of H-1 through H-6}}$$

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

4.B. 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 to Step 2.

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

   Note: Concentrate the cells by centrifuging and removing medium if the pool of cells is less than 100,000 cells/ml.

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

4. Create a spectrum of viability by blending sonicated and untreated 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	µl Sonicated	µl Untreated
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

4.B. Determining Assay Sensitivity, Method 2 (continued)

5. After mixing each blend by gently vortexing, pipet 100µl of each blend into 8 replicate wells of a 96-well plate. Add the 100% viable cells to column 1, 95% viable to column 2, etc. Add cell culture medium only to column 10 to serve as a no-cell control.

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

   Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate longer than 3 hours, and be sure to shield plates from ambient light.

7. Measure resulting fluorescence with a fluorometer (380–400nm$_{Ex}$/505nm$_{Em}$).

   Note: You may need to adjust instrument gains (applied photomultiplier tube energy).

8. Calculate the practical sensitivity for your cell type by making a signal-to-noise calculation for each blend of cell viability (X = 95, 90%, etc.).

$$\text{Viability S:N} = \frac{\text{(Average 100\% – Average X\%)}}{\text{Standard Deviation of 0\% (viable cells)}}$$

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

4.C. Example Viability Assay Protocol

1. Set up 96-well assay plates containing cells in culture medium at desired density.

2. Add test compounds and vehicle controls to 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 CellTiter-Fluor™ Reagent in an equal volume (100µl per well) to all wells, mix briefly by orbital shaking, then incubate for at least 30 minutes at 37°C.

   Note: Longer incubations may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours, and be sure to shield plates from ambient light.

5. Measure resulting fluorescence using a fluorometer (380–400nm$_{Ex}$/505nm$_{Em}$).

   Note: You may need to adjust instrument gains (applied photomultiplier tube energy).

4.D. Example Multiplex Assay Protocol (with luminescent caspase assay)

1. Set up 96-well assay plates containing cells in culture medium at the desired density.
2. Add test compounds and vehicle controls to 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.

   Note: Caspase activation is a transient event dictated by compound potency and cell cycle susceptibility. Time course experiments are often useful for defining peak caspase activity and cytotoxicity.
4. Add 20µl of CellTiter-Fluor™ Reagent (prepared as 10µl substrate in 2ml Assay Buffer) to all wells, 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. However, do not incubate longer than 3 hours, and be sure to shield plates from ambient light.
5. Measure resulting fluorescence using a fluorometer (380–400nm$_{Ex}$/505nm$_{Em}$).

   Note: You may need to adjust instrument gains (applied photomultiplier tube energy).
6. Add an equal volume of Caspase-Glo® 3/7 Reagent prepared as described in Technical Bulletin #TB323 to wells (100–120µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

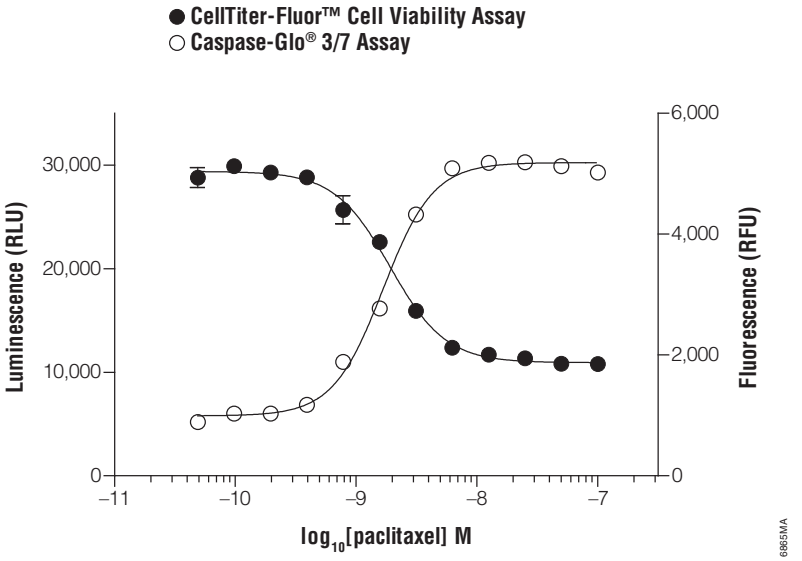

Figure 5. Multiplex of CellTiter-Fluor™ Assay and Caspase-Glo® 3/7 Assay. The CellTiter-Fluor™ Reagent was added to wells and viability measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent was added and luminescence measured after a 30-minute incubation (10,000 cells/well).

4.E. Recommended Controls

No-Cell Control: Set up triplicate wells without cells to serve as a 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.

Optional Test Compound Control: Set up triplicate wells without cells but containing the vehicle and test compound to test for possible interference with the assay chemistry.

Positive Control for Viability: Set up triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system.

5. General Considerations

Optical Filters and Instrumentation: Fluorogenic dyes exhibit distinct absorption (excitation) and emission profiles when a light energy source is applied. Most fluorometers or multimode instruments contain optical band-pass filters that restrict the wavelengths of light used to excite a fluorophore and the wavelengths passing through to the detector. Note that deviation from the optimal filter set recommendations (Figure 6) may adversely affect assay sensitivity and performance.

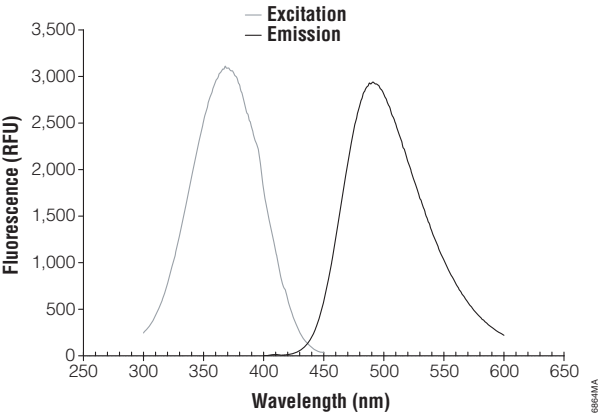

Figure 6. Optimal excitation and emission spectra for AFC.

Background Fluorescence and Inherent Serum Activity: Tissue culture medium that is supplemented with animal serum may contain detectable levels of the protease marker used to measure live-cells. This protease activity may vary among different lots of serum. To correct for variability, determine background fluorescence using samples containing medium plus serum without cells.

Temperature: The generation of fluorescent product is proportional to the live-cell protease activity. The activity of this protease is influenced by temperature.

For best results, we recommend incubating at a constant controlled temperature to ensure uniformity across the plate. After adding reagent and briefly mixing, we suggest one of two options:

1. 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 resulting from thermal gradients.

2. At room temperature with or without orbital shaking.

   Note: Assays performed at room temperature may require more than 30 minutes of incubation for optimal sensitivity. However, do not incubate longer than 3 hours.

Assay Controls: In addition to a no-cell control to establish background fluorescence, we recommend including a maximum viability (untreated cells) and maximum cytotoxicity control in the experimental design. The maximum viability control is established by adding vehicle only (used to deliver the test compound to test wells). In most cases, this consists of a buffer system or medium and the equivalent amount of solvent added with the test compound. The maximum cytotoxicity control can be determined using a compound that causes 100% cytotoxicity or a lytic compound added to compromise viability (digitonin). See Section 4.A.

Viability Marker Half-Life: The activity of the protease marker found has no half-life in viable cells. Viable cells will process the substrate to liberate the AFC fluorophore. However, when cells lose membrane integrity, the protease activity declines very quickly. Therefore enzymatic instability of the live-cell protease outside of viable cells establishes GF-AFC as a good marker for cell viability.

Light Sensitivity: Although the GF-AFC Substrate demonstrates good general photostability, the liberated AFC fluorophore (after contact with protease) 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 Substrate is introduced into the test well using an optimized buffer system that mitigates differences in pH from treatment. In addition, the buffer system supports protease activity in a host of different culture media with varying osmolarity. With the exception of media formulations with either very high serum content or phenol red indicator, no substantial performance differences will be observed among media.

6. References

1. 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.

2. 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.

7. Related Products

Cell Viability and CytotoxicityAssays

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
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® 2 Assay	10ml	G0940
Caspase-Glo® 6 Assay	10ml	G0970
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Apo-ONE® Homogeneous Caspase 3/7 Assay	10ml	G7790

Additional Sizes Available.

Reporter Gene Assays

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

Additional Sizes Available.

[a]Patent Pending.