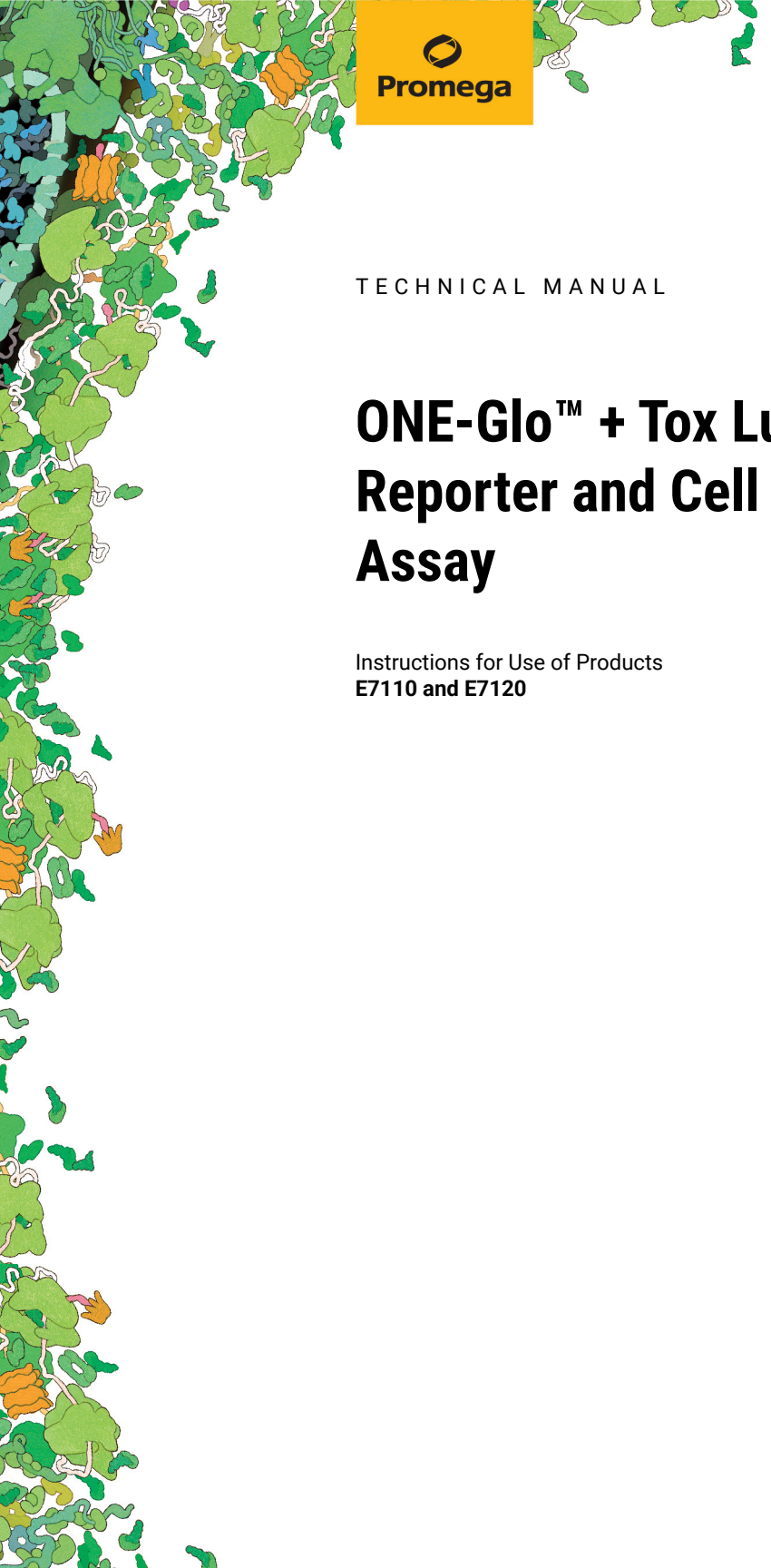

TECHNICAL MANUAL

# ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay

Instructions for Use of Products
**E7110 and E7120**

Revised 11/26
TM356

Promega

# ONE-Glo™ + Tox Luciferase Reporter and Cell Viability 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 ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay combines assay chemistries to better understand firefly luciferase reporter gene expression in the context of cell viability. The assay uses a two-step, addition-only process to make these measurements in a single well of a plate, negating the need to run parallel assays.

The first part of the assay is a nonlytic fluorescence assay (CellTiter-Fluor™ Cell Viability Assay) that measures the relative number of live cells in a culture population after experimental manipulation. The CellTiter-Fluor™ Assay measures the conserved and constitutive protease activity of cathepsin C within live cells and therefore serves as a marker of cell viability (1,2). The live-cell-protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC). The substrate enters intact cells where it is cleaved by the live-cell protease to generate a fluorescent signal proportional to the number of living cells (Figure 1). This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Fluorescence of the free AFC fluorophore is measured with a microplate reader or CCD imager using an excitation wavelength of 380–400nm and emission wavelength of 505nm.

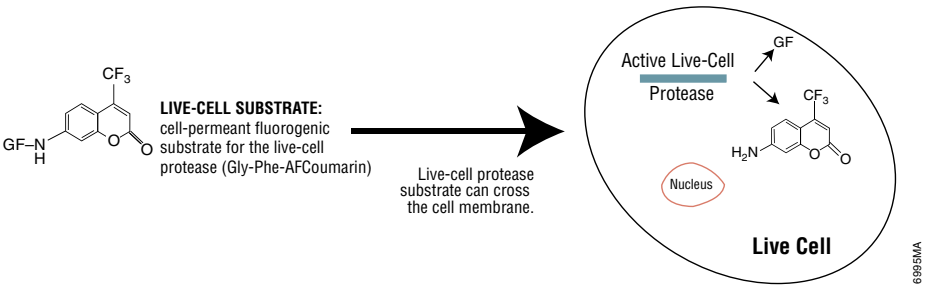


**Figure 1. CellTiter-Fluor™ Cell Viability Assay chemistry.** The cell-permeant substrate enters the cell, where it is cleaved by the live-cell protease to produce the fluorescent AFC. The live-cell protease is labile in membrane-compromised cells and cannot cleave the substrate.

The second part of the assay uses the ONE-Glo™ Luciferase Assay System to quantify firefly luciferase reporter gene expression from cells made to express this reporter enzyme (Figure 2). The ONE-Glo™ Luciferase Assay Buffer and ONE-Glo™ Luciferase Assay Substrate, provided with this system, are combined to form the ONE-Glo™ Reagent. Ideally suited for high- and ultrahigh-throughput applications, the ONE-Glo™ Assay contains a new fluoroluciferin substrate, resulting in a reagent that is more stable, more tolerant to sample components, and has less odor than standard luciferase assay reagents. Luminescence is measured with a microplate reader or CCD imager.

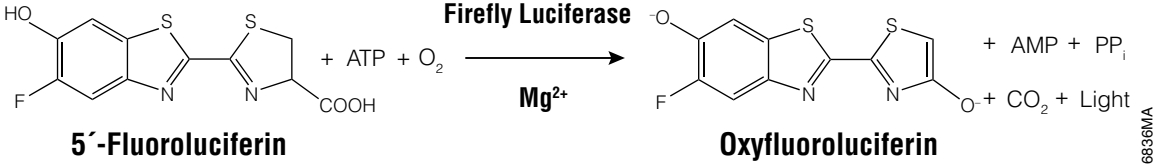


**Figure 2. The luciferase reaction.** Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of magnesium, ATP and molecular oxygen.

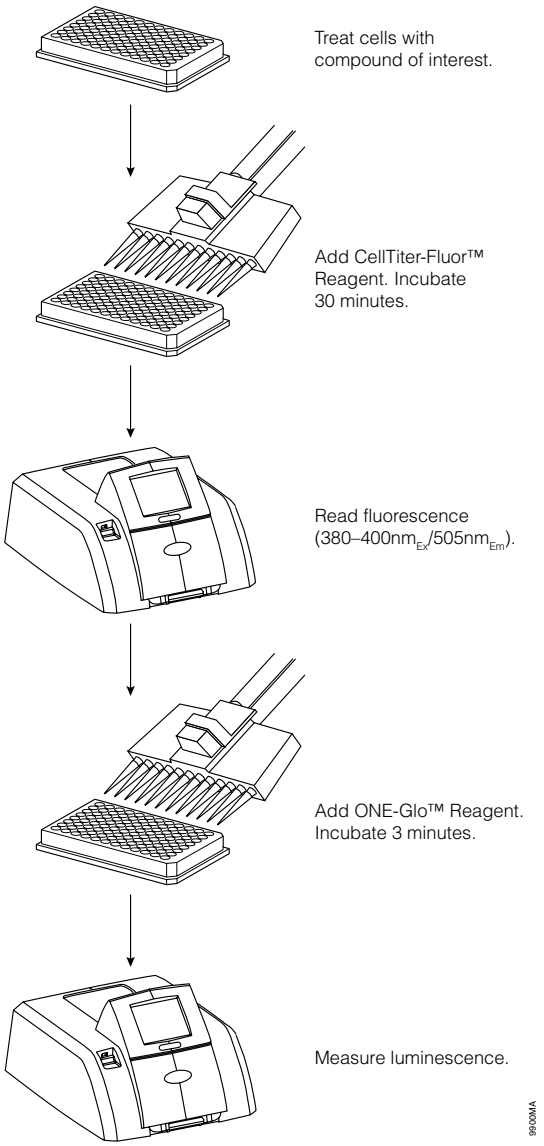


**Figure 3. Schematic diagram of the ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay.**

## 2. Product Components and Storage Conditions

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay** | **1 plate** | **E7110** |

For Research Use. Cat.# E7110 contains sufficient reagents for 100 assays in a 96-well-plate format or 400 assays in a 384-well-plate format. Includes:

- 1 × 10ml Assay Buffer
- 1 × 10µl GF-AFC Substrate
- 1 × 10ml ONE-Glo™ Luciferase Assay Buffer
- 1 vial ONE-Glo™ Luciferase Assay Substrate (lyophilized)

| PRODUCT | SIZE | CAT.# |
|---|---|---|
| **ONE-Glo™ + Tox Luciferase Reporter and Cell Viability Assay** | **10 plates** | **E7120** |

For Research Use. Cat.# E7120 contains sufficient reagents for 1,000 assays in a 96-well-plate format or 4,000 assays in a 384-well-plate format. Includes:

- 1 × 50ml Assay Buffer
- 2 × 50µl GF-AFC Substrate
- 1 × 100ml ONE-Glo™ Luciferase Assay Buffer
- 1 vial ONE-Glo™ Luciferase Assay Substrate (lyophilized)

**Storage Conditions for CellTiter-Fluor™ Assay:** Store the Assay Buffer and GF-AFC Substrate at –30°C to –10°C. See the kit label for expiration date.

**Storage Conditions for the ONE-Glo™ Luciferase Assay System:** Store the ONE-Glo™ Luciferase Assay System components at –30°C to –10°C. The substrate also can be stored at room temperature for up to 3 weeks or at +2°C to +10°C for up to 3 months with only a 12% decrease in functionality. The buffer also can be stored at room temperature for up to 3 months with approximately a 10% change in reagent functionality. For convenience the buffer label contains a space on which to record the date that the buffer is transferred to room temperature. Store the buffer at room temperature to prevent the need for temperature equilibration when the reagent is reconstituted.

## 3. Reagent Preparation, Stability and Storage Conditions

### 3.A. CellTiter-Fluor™ Cell Viability Assay

1. Completely thaw the GF-AFC Substrate and Assay Buffer in a 37°C water bath. Vortex the GF-AFC Substrate to ensure homogeneity, then briefly centrifuge for complete substrate volume recovery.
2. **Option 1:** Prepare 5X CellTiter-Fluor™ Reagent by transferring 10µl of GF-AFC Substrate to 2ml of Assay Buffer. Mix by vortexing the contents until the substrate is thoroughly dissolved. See Section 4.A for more information.

   **Option 2:** Prepare 2X CellTiter-Fluor™ Reagent by transferring 10µl of GF-AFC Substrate to 10ml of Assay Buffer. Mix by vortexing the contents until the substrate is thoroughly dissolved. See Section 4.A for more information.

   **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.

**Reagent Storage Conditions:** The CellTiter-Fluor™ 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.

### 3.B. ONE-Glo™ Luciferase Assay System

1. Completely thaw the ONE-Glo™ Buffer.
2. Transfer the contents of one bottle of ONE-Glo™ Buffer to one bottle of ONE-Glo™ Substrate. Mix by inversion until the substrate is thoroughly dissolved. This may require multiple inversions.

**Notes:**

a. Since luciferase activity is temperature-dependent, the temperature of the ONE-Glo™ Reagent should be kept constant while quantitating luminescence. This is most easily accomplished by using reagent that is equilibrated to room temperature. Equilibration of the reagent prior to use is unnecessary when the buffer is stored at room temperature.

b. If the reagent is stored at 4°C or frozen after reconstitution, it must be warmed or thawed at temperatures below 25°C to ensure optimal performance. The most convenient and effective method for thawing or equilibrating cold reagent is by placing the reagent in a water bath at room temperature. Mix well after thawing.

c. For maximum reproducibility, equilibrate cultured cells to room temperature before adding the reagent.

**Reagent Stability and Storage Conditions:** Approximate stability of ONE-Glo™ Reagent after reconstitution: 18% loss of luminescence over 24 hours at room temperature and 12% loss over 5 days at 4°C. No change in functionality was measured after 9 weeks at –20°C. The reagent can be subjected to up to 10 freeze-thaw cycles with no effect on functionality.

## 4. Protocol

### Materials to Be Supplied by the User

- 96-, 384-, or 1,536-well white opaque-walled tissue culture plates compatible with your detection instrumentation
- multichannel pipettor or liquid-dispensing robot
- reagent reservoirs
- fluorescence plate reader or CCD imager with filter sets for AFC (380–400nm$_{Ex}$/505nm$_{Em}$)
- luminescence plate reader or CCD imager
- orbital plate shaker or electromagnetic shaker
- compound known to cause 100% cytotoxicity or lytic detergent (e.g., 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 in Section 5.B. If you do not need to determine assay sensitivity for your cells, proceed to Section 4.A.

### 4.A. Recommended Assay Volumes

The ONE-Glo™ + Tox Assay can be scaled to meet throughput needs. Table 1 contains example volumes for cell culture and reagent addition in a variety of multiwell plate formats. The CellTiter-Fluor™ Reagent can be dispensed as a 2X or 5X reagent as outlined in Section 3.A followed by ONE-Glo™ Reagent volumes added to the same well as outlined in Table 1. The volumes listed in parentheses are for the option to add the 2X CellTiter-Fluor™ Reagent. Both concentrations of CellTiter-Fluor™ Reagent are equivalent in terms of assay performance. The assay protocol chosen often is dictated by volumes that are compatible with multichannel pipettors or liquid handling automation. In that regard, the 2X protocol is recommended for higher density assay plates for more accurate volume additions and better well mixing.

**Table 1. Suggested Volumes of Cell Culture and Reagent Volumes.** Volumes listed in parentheses are recommended for adding the 2X CellTiter-Fluor™ Reagent to the well.

| | 96-Well Plate | Half-Area 96-Well Plate | 384-Well Plate | Low-Volume 384-Well Plate | 1,536-Well Plate |
|---|---|---|---|---|---|
| **Cell culture, including treatment** | 100µl (50µl) | 50µl (25µl) | 25µl (20µl) | (5µl) | (2µl) |
| **CellTiter-Fluor™ Reagent** | 20µl (50µl) | 10µl (25µl) | 5µl (20µl) | (5µl) | (2µl) |
| **ONE-Glo™ Reagent** | 100µl (100µl) | 50µl (50µl) | 25µl (40µl) | (10µl) | (4µl) |

**Note:** Following the 2X protocol may require more ONE-Glo™ Assay Reagent than what is included in the kit.

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### 4.B. Example 96-Well Protocol

1. Set up 96-well assay plates containing cells capable of expressing firefly luciferase in culture medium at the desired density. Assay plates should be compatible with fluorescence and luminescence measurements, preferably with white opaque-walled wells.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 100µl in each well.
3. Culture cells for the desired test exposure period and under conditions resulting in luciferase reporter expression.
4. Add 20µl of CellTiter-Fluor™ Reagent (prepared as 5X solution using 10µl of GF-AFC Substrate in 2ml of Assay Buffer) to all wells, and mix briefly with an orbital (300–500rpm for ~30 seconds) or electromagnetic shaker. 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 100µl of ONE-Glo™ Reagent to each well, incubate for 3 minutes, then measure luminescence using a luminometer.

### 4.C. Example 384-Well Protocol

1. Set up 384-well assay plates containing cells capable of expressing firefly luciferase in culture medium at the desired density. Assay plates should be compatible with fluorescence and luminescence measurements, preferably with white opaque-walled wells.
2. Add test compounds and vehicle controls to appropriate wells so that the final volume is 25µl in each well.
3. Culture cells for the desired test exposure period and under conditions resulting in luciferase reporter expression.
4. Add 5µl of CellTiter-Fluor™ Reagent (prepared as 5X solution with 10µl of GF-AFC Substrate in 2ml of Assay Buffer) to all wells, and mix briefly with an orbital (1,000–1,200rpm for ~30 seconds) or electromagnetic shaker. 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 or CCD imager (380–400nm$_{Ex}$/505nm$_{Em}$).

   **Note:** You may need to adjust instrument gains (applied photomultiplier tube energy).
6. Add 25µl of ONE-Glo™ Reagent to each well, incubate for 3 minutes, then measure luminescence using a luminometer or CCD imager.

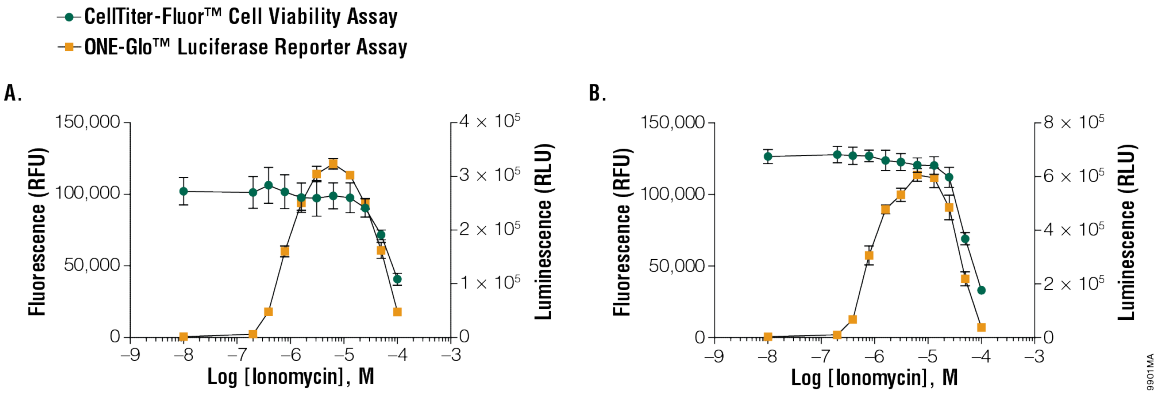


**Figure 4. Ionomycin titration in standard 96- and 384-well plate formats.** 1 × $10^4$ (96-well plate; **Panel A**) or 5 × $10^3$ (384-well plate; **Panel B**) cells expressing NFAT response element were treated with serial titrations of ionomycin in the presence of PMA for 6 hours following the example protocols in Section 4.B and 4.C. At specific concentrations, ionomycin and PMA work cooperatively to stimulate NFAT-dependent gene expression. However, higher concentrations of ionomycin result in cytotoxicity seen as a decrease in viability (fluorescence). A decrease in reporter expression (luciferase) activity is also observed due to the increase in cytotoxicity.

## 5. General Considerations for CellTiter-Fluor™ Cell Viability Assay

### 5.A. 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 Cytotoxicity:** Set up triplicate wells containing cells treated with a compound known to be toxic to the cells used in your model system.

### 5.B. Determining Assay Sensitivity of the CellTiter-Fluor™ Cell Viability Assay

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.

**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 2).

**Table 2. 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 | | | | | | | | | | | |

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 cells/well in row A to 156 cells/well in row G. Row H will serve as the no-cell, background control.

6. Dilute digitonin to 300µg/ml in water. Using a multichannel pipette, 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 (prepared as a 2X reagent by adding 10µl of GF-AFC Substrate to 10ml of Assay Buffer) 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).

**5.B. Determining Assay Sensitivity of the CellTiter-Fluor™ Cell Viability Assay (continued)**

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.).

$$\text{Viability S:N} = \frac{\text{(Average Untreated – Average Treated)}}{\text{Standard Deviation of H1 through H6}}$$

**Note:** The practical level of assay sensitivity for the assay is a signal-to-noise ratio of greater than three standard deviations (derived from reference 1). Ensure that the signal from your cells is above the background of the assay plate (wells H1–H6).

**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 3.

**Table 3. 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 |
| 9 | 1,000 | 0 |

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 (prepared as a 2X reagent by adding 10µl of GF-AFC Substrate to 10ml of Assay Buffer) 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 three standard deviations (derived from reference 3).

### 5.C. 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 5) may adversely affect assay sensitivity and performance.

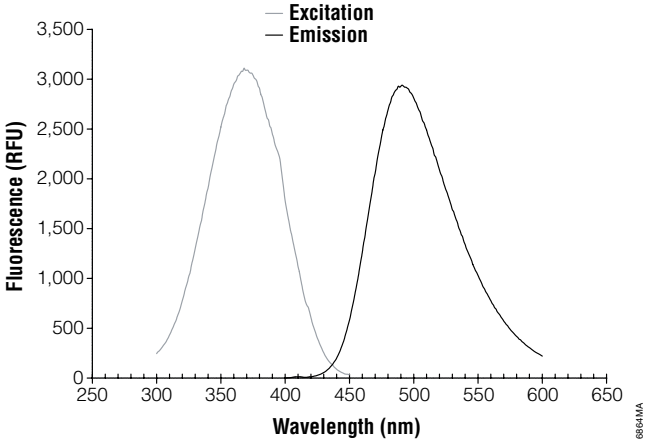


**Figure 5. Optimal excitation and emission spectra for AFC.**

### 5.D. 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.

### 5.E. Temperature

The generation of fluorescent product in the CellTiter-Fluor™ Cell Viability Assay 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. 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 resulting from 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 for optimal sensitivity. However, do not incubate longer than 3 hours.

## 6. General Consideration for the ONE-Glo™ Luciferase Assay Reagent

### 6.A. Recommended Controls

The luminescence signal is affected by assay conditions; results should be compared only between samples measured using the same medium/serum combinations. For analysis of multiple plates, the greatest accuracy can be obtained by incorporation of a common control sample in each plate. By this method, luminescence measurements of each plate can be normalized to the control contained within the same plate. This allows for the correction of small variations in luminescence that can occur over time or due to other variables such as temperature.

### 6.B. Effect of Medium on Luminescence

Like other homogeneous luciferase assays, half of the reaction volume for ONE-Glo™ Assay reactions is mammalian tissue culture medium. The ONE-Glo™ Reagent is designed to work well with a variety of common media. However, differences between media can affect the intensity and duration of the luminescent signal. Differences in media or between different manufacturers or lots of the same medium make incorporating controls in each batch of plates advisable.

## 6.C. Assay Background

To achieve linear assay performance at low light levels, the background luminescence must be subtracted from all readings. No background is produced by the ONE-Glo™ Reagent or by mammalian cells lacking the luciferase gene, but background luminescence is a characteristic of luminometer performance. Some instruments also require verification of linear response at high light levels (consult the instrument manual).

## 7. References


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## 8. Additional Resources


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2. Schagat, T. and Kopish, K. Optimize transfection of cultured cells. [Internet] 2009. Available from: **www.promega.com/pubs/tpub_020.htm**
3. *CellTiter-Fluor™ Cell Viability Assay Technical Bulletin* #TB371, Promega Corporation.
4. *ONE-Glo™ Luciferase Assay System Technical Manual* #TM292, Promega Corporation.
5. *FuGENE® HD Transfection Reagent Technical Manual* #TM328, Promega Corporation.


## 9. Summary of Changes

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

1. Updated Sections 1 and 7.
2. Removed the Related Products section.
3. Removed patent statements.
4. Replaced the cover page and document font.
5. Made minor text edits.

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