Technical Bulletin

CytoTox-Fluor™ Cytotoxicity Assay

INSTRUCTIONS FOR USE OF PRODUCTS G9260, G9261 AND G9262.

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1. Description

The CytoTox-Fluor™ Cytotoxicity Assay[a] is a single-reagent-addition, homogeneous, fluorescent assay that measures the relative number of dead cells in cell populations. The CytoTox-Fluor™ Assay measures a distinct protease activity associated with cytotoxicity. The assay uses a fluorogenic peptide substrate (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) to measure "dead-cell protease activity," which has been released from cells that have lost membrane integrity. The bis-AAF-R110 Substrate cannot cross the intact membrane of live cells and therefore gives no signal from live cells.

The CytoTox-Fluor™ Assay is designed to accommodate downstream multiplexing with any Promega luminescent assay or spectrally distinct fluorescent assay methods, such as assays measuring caspase activation, reporter expression or orthogonal measures of viability.

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Assay Advantages

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

Get More Data from Every Well: Multiplex the CytoTox-Fluor™ Assay with any Promega luminescent assay (apoptosis determination, reporter gene, or protease activity assays).

Normalize Downstream Multiplex Data for Cytotoxicity: Data normalization for dead-cell number makes results more comparable well-to-well, plate-to-plate, day-to-day.

2. Product Components and Storage Conditions

Product	Size	Cat.#
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260

G9260 contains sufficient reagents for 100 assays at 100µl/assay in a 96-well plate format or 400 assays at 25µl/assay in a 394-well plate format. Includes:

- 1 × 10ml Assay Buffer (Part# G610A)
- 1 × 10µl bis-AAF-R110 Substrate (100mM in DMSO)

Product	Size	Cat.#
CytoTox-Fluor™ Cytotoxicity Assay	5 × 10ml	G9261

G9261 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 (Part# G610A)
- 5 × 10µl bis-AAF-R110 Substrate (100mM in DMSO)

Product	Size	Cat.#
CytoTox-Fluor™ Cytotoxicity Assay	2 × 50ml	G9262

G9262 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 (Part# G610B)
- 2 × 50µl bis-AAF-R110 Substrate (100mM in DMSO)

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

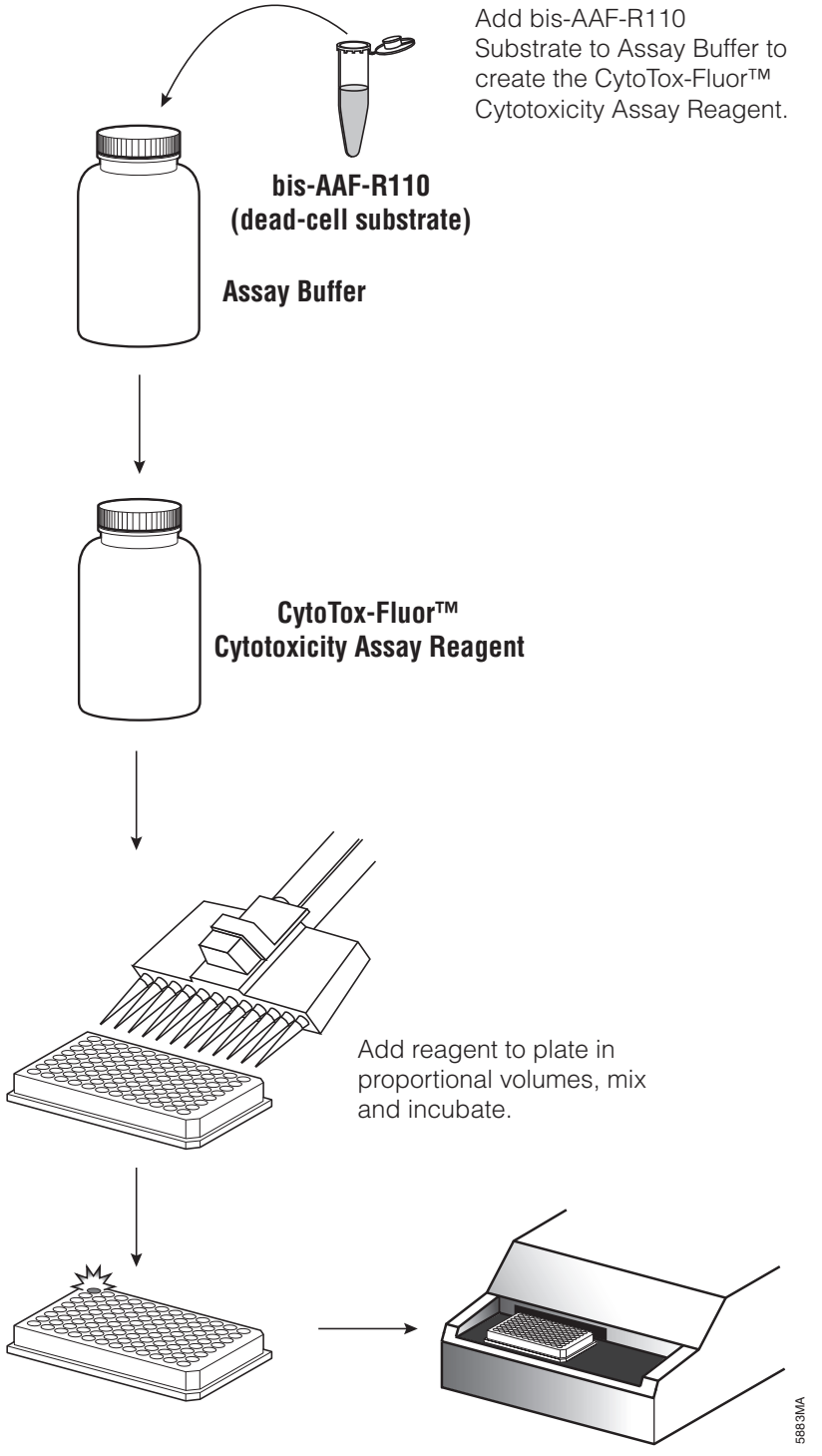

Figure 1. Schematic diagram of the CytoTox-Fluor™ Cytotoxicity Assay.

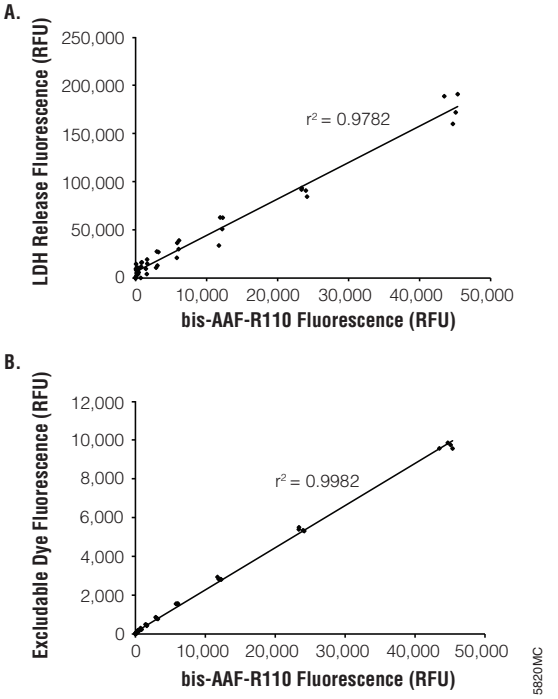

Figure 2. The CytoTox-Fluor™ Cytotoxicity Assay shows strong correlation with established methods for measuring cytotoxicity. Panel A. The bis-AAF-R110 Substrate signal from serial dilutions of dead cells plotted against results from the CytoTox-ONE™ Assay, which measures LDH release. **Panel B.** The bis-AAF-R110 Substrate signal from serial dilutions of dead cells plotted against results achieved using ethidium homodimer.

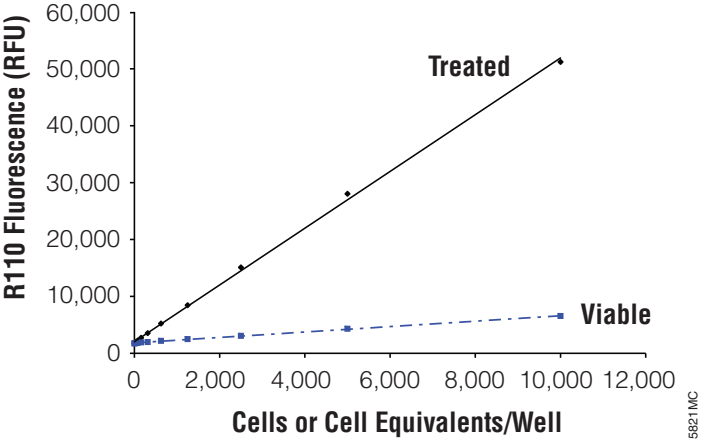

Figure 3. The CytoTox-Fluor™ Cytotoxicity Assay signals derived from viable cells (untreated) or lysed cells (treated) are proportional to cell number.

3. Reagent Preparation and Storage

1. Thaw the CytoTox-Fluor™ Cytotoxicity Assay components in a 37°C water bath.
2. Transfer the bis-AAF-R110 Substrate (10µl for Cat.# G9260 and G9261; 50µl for Cat.# G9262) into the Assay Buffer container (10ml for Cat.# G9260 and G9261; 50ml for Cat.# G9262) for a 2X Reagent. Mix by vortexing the contents until the substrates are thoroughly dissolved to create the reagent.

   Note: The CytoTox-Fluor™ Reagent may be scaled to accommodate the volumes required for downstream multiplexes. To do this, use 1/10 the volume of buffer when you prepare the reagent (i.e., 10µl of the bis-AAF-R110 Substrate in 1ml of Assay Buffer). Add 1/10 volume of reagent (10µl) to the assay.

   Storage: The CytoTox-Fluor™ Cytotoxicity Reagent should be used within 24 hours if stored at room temperature. Unused bis-AAF-R110 Substrate and Assay Buffer can be stored at 4°C for up to 7 days with no appreciable loss of activity.

4. Protocols for CytoTox-Fluor™ Cytotoxicity 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 rhodamine-110 ($485nm_{Ex}/520_{Em}$)
- orbital plate shaker
- positive control cytotoxicity compound or lytic detergent (digitonin, Calbiochem Cat.# 300410 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: Concentration by centrifugation may be necessary 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) into all wells of row A and B in a 96-well plate (see Table 1).

4.A. Determining Assay Sensitivity, Method 1 (continued)

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 a 100µl volume from row B to row C. Repeat mixing and transfer 100µl from row C to row D, and 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	No Cells/Well											
	untreated samples						**treated samples**					

6. Dilute digitonin to 300µg/ml in water. Using a multichannel pipet, carefully add 10µl of the solution 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 CytoTox-Fluor™ Cytotoxicity Assay Reagent to all wells, mix briefly by orbital shaking (to ensure homogeneity) 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.**
8. Measure resulting fluorescence with fluorometer ($485nm_{Ex}/520nm_{Em}$)

   Note: Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.
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.).

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

   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.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 them by dilution to 100,000 viable cells/ml in at least 20ml of fresh medium.

   Note: Centrifugation concentration may be necessary 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 post-sonication morphological examination) to rupture cell membrane integrity and to simulate a 100% cytotoxic 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 Eppendorf tubes as described in Table 2.

Table 2. Spectrum of Viability Generated 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

5. After mixing each blend by gently vortexing, pipet 100µl of each blend into 8 replicate wells of 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 no-cell control.

6. Add CytoTox-Fluor™ Cytotoxicity Assay 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.**

7. Measure resulting fluorescence with a fluorometer (485nm$_{Ex}$/520nm$_{Em}$).

   Note: Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.

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

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))

$$\text{Cytotoxicity S:N} = \frac{(\text{Average X\%} - \text{Average 100\%})}{\text{Std Dev of 100\%}}$$

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).

4.C. Example Cytotoxicity 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 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 CytoTox-Fluor™ Cytotoxicity Assay 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.**
5. Measure resulting fluorescence using a fluorometer ($485nm_{Ex}/520nm_{Em}$).

   Note: Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.

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

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 the final volume in the well is 100µl in each well (25µl for a 384-well plate).
3. Culture cells for the desired test exposure period.
4. Add 10µl CytoTox-Fluor™ Cytotoxicity Assay Reagent **(prepared as 10µl substrate in 1ml 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.**
5. Measure resulting fluorescence using fluorometer ($485nm_{Ex}/520nm_{Em}$).

   Note: Adjustment of instrument gains (applied photomultiplier tube energy) may be necessary.
6. Add an equal volume of Caspase-Glo® 3/7 Reagent to wells (100–110µl per well), incubate for 30 minutes, then measure luminescence using a luminometer.

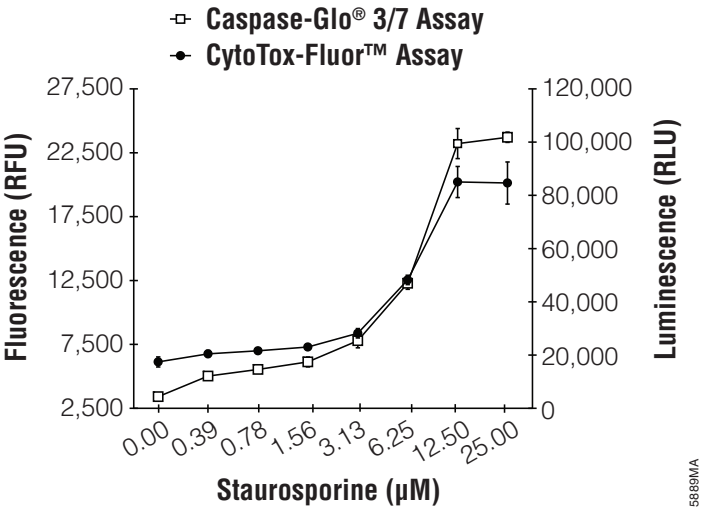

Figure 4. CytoTox-Fluor™ Assay multiplexed with Caspase-Glo® 3/7 Assay. The CytoTox-Fluor™ Assay Reagent is added to wells and cytotoxicity measured after incubation for 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent is added and luminescence measured after a 30-minute incubation.

4.E. 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.

Optional Test Compound Control: Set up triplicate wells without cells 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. 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 measurement. The quantity of this protease activity may vary among different lots of serum. To correct for variability, background fluorescence should be determined using samples containing medium plus serum without cells.

5. General Considerations (continued)

Temperature

The generation of fluorescent product is proportional to the protease activity of the marker associated with cytotoxicity. 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 reagent addition and brief mixing, we suggest one of two methods:

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. However, **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 cells (maximum viability) and positive (maximum cytotoxicity) control in the experimental design. The maximum viability control is established by the addition of 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 cytotoxicity or a lytic compound added to compromise viability (non-ionic or Zwitterionic detergents). See Section 4.A.

Cytotoxicity Marker Half-Life

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

Light Sensitivity

Although the bis-AAF-R110 Substrate demonstrates good general photostability, the liberated fluors (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 bis-AAF-R110 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. Reference

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

7. Related Products

Apoptosis Assays

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

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.

Protease Assays

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml*	G8501
DPPIV-Glo™ Protease Assay	10ml*	G8350
Proteasome-Glo™ Cell-Based Assay	10ml*	G8660
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601

*Additional Sizes Available.

7. Related Products (continued)

Cell Viability Assays

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

ADME/Tox Assays

Product	Size	Cat.#
P450-Glo™ CYP1A1 Assay	50ml	V8752
P450-Glo™ CYP1B1 Assay	50ml	V8762
P450-Glo™ CYP1A2 Assay	50ml	V8772
P450-Glo™ CYP2C8 Assay	50ml	V8782
P450-Glo™ CYP2C9 Assay	50ml	V8792
P450-Glo™ CYP3A4 Assay	50ml	V8802
P450-Glo™ CYP3A7 Assay	50ml	V8812
P450-Glo™ CYP2D6 Assay	50ml	V8892
P450-Glo™ CYP2C19 Assay	50ml	V8882
MAO-Glo™ Assay	50ml	V1402

*Additional Sizes Available.

[a] Patent Pending.