TECHNICAL MANUAL

CellTox™ Green Cytotoxicity Assay

Instructions for Use of Products
G8741, G8742, G8743 and G8731

Revised 11/13
TM375

CellTox™ Green Cytotoxicity Assay

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The CellTox™ Green Cytotoxicity Assay[a] measures changes in membrane integrity that occur as a result of cell death. The assay is intended for assessing cytotoxicity in cell culture after experimental manipulation. The assay system uses a proprietary asymmetric cyanine dye (1) that is excluded from viable cells but preferentially stains the dead cells' DNA (Figure 1). When the dye binds DNA in compromised cells, the dye's fluorescent properties are substantially enhanced. Viable cells produce no appreciable increases in fluorescence. Therefore, the fluorescent signal produced by the dye binding to the dead-cell DNA is proportional to cytotoxicity (Figure 2).

The CellTox™ Green Dye is well tolerated by a wide variety of cell types and is essentially nontoxic. The dye can be diluted in culture medium and delivered directly to cells at seeding or at dosing, allowing "no-step" kinetic measures of cytotoxicity (Figure 3). The dye also can be diluted in assay buffer and delivered to cells as a conventional endpoint measure after an exposure period (Figure 3).

The CellTox™ Green Cytotoxicity Assay offers additional utility in that the assay can be multiplexed with other spectrally distinct measures of cell health to provide mechanistic information relating to cytotoxicity.

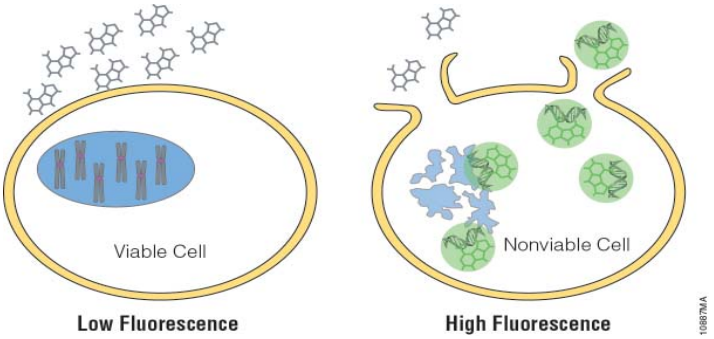

Figure 1. CellTox™ Green Dye binds DNA of cells with impaired membrane integrity.

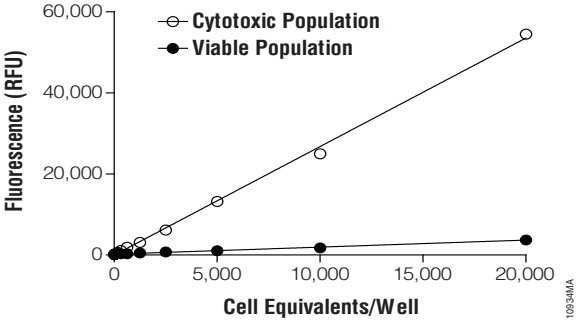

Figure 2. CellTox™ Green Dye fluorescence is proportional to dead-cell number. K562 cells were adjusted to 200K/ml in RPMI 1640 + 10% FBS then divided into two volumes. One volume was treated with 30µg/ml digitonin, the other untreated. Both were twofold serially diluted in RPMI + 10% FBS. CellTox™ Green was made as a 2X reagent and added. Fluorescence was measured using the BMG PolarStar after 15 minutes of incubation. Ex: 485nm, Em: 520nm.

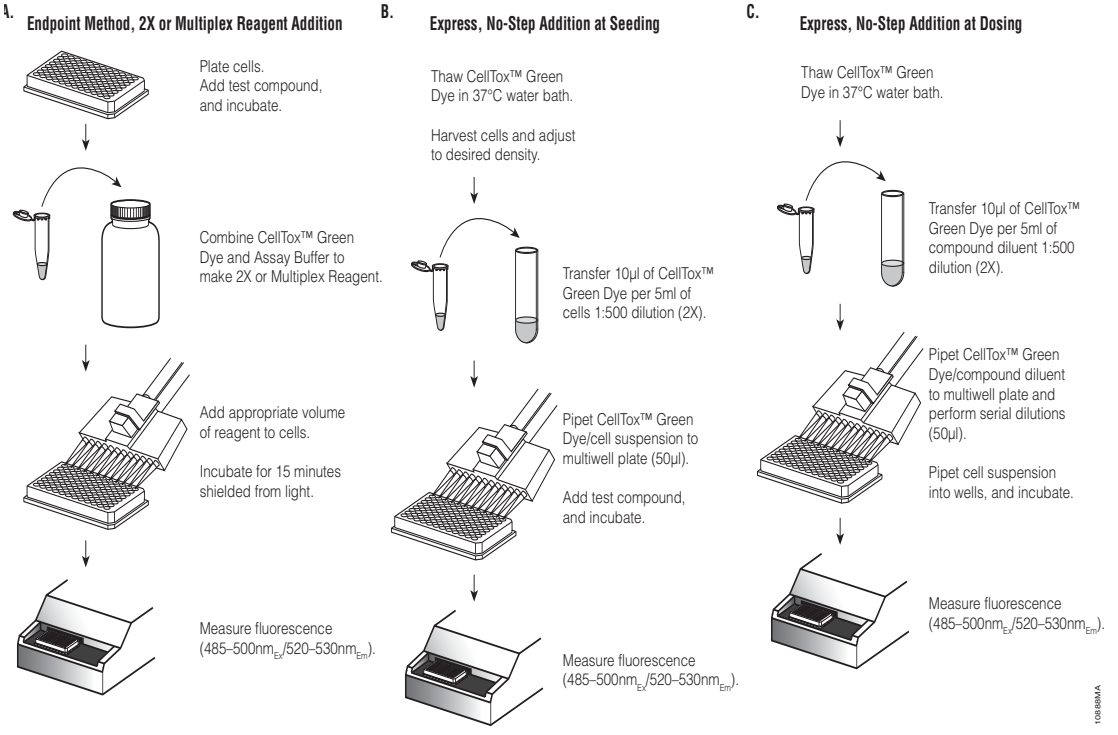

Figure 3. Schematic diagram of methods for delivering the CellTox™ Green Dye. Diagram shows the protocol for a 96-well plate.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CellTox™ Green Cytotoxicity Assay	**10ml**	**G8741**

Cat.# G8741 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 following the Endpoint Assay Protocol, 2X Reagent Addition. Includes:

- 20µl CellTox™ Green Dye, 1,000X
- 10ml Assay Buffer
- 0.5ml Lysis Solution

PRODUCT	SIZE	CAT.#
CellTox™ Green Cytotoxicity Assay	**50ml**	**G8742**

Cat.# G8742 contains sufficient reagents for 500 assays at 100µl/assay in a 96-well plate format or 2,000 assays at 25µl/assay in a 384-well plate format following the Endpoint Assay Protocol, 2X Reagent Addition. Includes:

- 5 × 20µl CellTox™ Green Dye, 1,000X
- 50ml Assay Buffer
- 0.5ml Lysis Solution

PRODUCT	SIZE	CAT.#
CellTox™ Green Cytotoxicity Assay	**100ml**	**G8743**

Cat.# G8743 contains sufficient reagents for 1,000 assays at 100µl/assay in a 96-well plate format or 4,000 assays at 25µl/assay in a 384-well plate format following the Endpoint Assay Protocol, 2X Reagent Addition. Includes:

- 200µl CellTox™ Green Dye, 1,000X
- 2 × 50ml Assay Buffer
- 0.5ml Lysis Solution

PRODUCT	SIZE	CAT.#
CellTox™ Green Express Cytotoxicity Assay	**200µl**	**G8731**

Cat.# G8731 contains sufficient reagents for 1,000 assays at 100µl of the CellTox™ Green Reagent in a 96-well plate format or 4,000 assays in a 384-well plate format when used at the recommended final dilution of 1:1,000 in the assay well. Includes:

- 1 × 200µl CellTox™ Green Dye, 1,000X

Storage Conditions: Store the CellTox™ Green Cytotoxicity Assay and CellTox™ Green Express Cytotoxicity Assay components at –20°C. See product label for expiration date.

Available Separately

PRODUCT	SIZE	CAT.#
Lysis Solution	5ml	G1821

3. Reagent Preparation and Storage

The CellTox™ Green Dye used in the CellTox™ Green Cytotoxicity Assay and CellTox™ Green Express Cytotoxicity Assay can be diluted and delivered to test wells by four different methods depending upon the desired format. In our hands, a final dilution of the CellTox™ Green Dye to 1:1,000 in the presence of cells has provided optimal performance. (Other concentrations [from 1:500 to 1:2,000] may be optimal for some cell types and/or medium compositions.) The Endpoint Method, 2X Reagent Addition, is intended for a standard endpoint cytotoxicity measurement. The Endpoint Method, Multiplex Reagent Addition, is intended for endpoint cytotoxicity measurements that will be multiplexed with other spectrally distinct assay chemistries. The Express, No-Step Addition formats are intended for kinetics studies of cytotoxicity involving multiple plate measurements over a time course. These No-Step formats also allow multiplexing opportunities with other assay chemistries at the completion of the time course.

3.A. Reagent Preparation for Endpoint Method, 2X Reagent Addition

1. Completely thaw the CellTox™ Green Cytotoxicity Assay components in a 37°C water bath. To ensure homogeneity, mix each component separately using a vortex mixer: the CellTox™ Green Dye, Assay Buffer and Lysis Solution. A brief centrifugation may be necessary to collect all of the liquid at the bottom of the tube.
2. To create the CellTox™ Green Reagent (2X), transfer CellTox™ Green Dye (20µl for Cat.# G8741 or 100µl for Cat.# G8743) to the Assay Buffer bottle provided (1:500 dilution). For CellTox™ Green Cytotoxicity Assay in the 50ml size (Cat.# G8742), determine the required reagent volume and transfer the Assay Buffer to a clean centrifuge of an appropriate size. Mix the combined Assay Buffer and CellTox™ Green Dye solution using a vortex mixer to ensure homogeneity. Protect the CellTox™ Green Reagent from light before use.

   Storage: We recommend preparing only the volume of CellTox™ Green Reagent (2X) required for an individual experiment; however, the CellTox™ Green Reagent (2X) can be stored at room temperature for 24 hours. Unused CellTox™ Green Reagent (2X) can be stored at 4°C for up to seven days with no appreciable change in performance.

3.B. Reagent Preparation for Endpoint Method, Multiplex Reagent (~5X) Addition (for multiplexing applications)

1. Completely thaw the CellTox™ Green Cytotoxicity Assay components in a 37°C water bath. Mix the CellTox™ Green Dye, Assay Buffer and Lysis Solution using a vortex mixer to ensure homogeneity. A brief centrifugation may be necessary to collect all of the liquid at the bottom of the tube.
2. Estimate the volume of CellTox™ Green Reagent (~5X) required. For each 2ml reagent estimated, transfer 2ml of Assay Buffer to a clean centrifuge tube of the appropriate size. Add 20µl of the CellTox™ Green Dye for each 2ml of dispensed Assay Buffer (1:100 dilution). Mix the combined Assay Buffer and CellTox™ Green Dye using a vortex mixer to ensure homogeneity. Shield the CellTox™ Green Reagent (~5X) from ambient light before use.

   Note: If additional dead volume is required to compensate for pipetting losses in a reagent reservoir, transfer 2.2ml of Assay Buffer to the centrifuge tube for each 20µl of dye. This higher buffer-to-dye ratio (relative to standard concentration) will not affect assay performance.

4. Validation Protocols for the CellTox™ Green Cytotoxicity Assay

Materials To Be Supplied by the User

- opaque-walled 96- or 384-well tissue culture plates compatible with fluorometer (clear- or solid-bottom)
- multichannel pipettor and tips or liquid-dispensing robot
- reagent reservoirs
- multiwell fluorescence plate reader with 485–510nm excitation source and 520–530nm emission filter or monochromator
- vortex mixer and orbital shaker
- cells and medium
- water bath
- hemocytometer and trypan blue

Note: If you have not performed this assay with your cell line previously, we recommend determining the assay sensitivity and linearity using your cells and one of the two methods described in Section 4.A or 4.B

4.A. Determining Linear Range and Sensitivity, Method 1

Note: This method is written for Endpoint Method, 2X Reagent Addition, but the resulting data are applicable to all of the assay formats.

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 hemocytometer, then adjust the cells to a concentration of 200,000 viable cells/ml in at least 4.0ml of fresh medium in a conical centrifuge tube.

   Note: Concentration by centrifugation may be necessary if the cell suspension is less than 200,000 viable cells/ml. Overall viability of less than 95% will increase background and decrease the assay signal window.

3. Divide the cell suspension created in Step 2 by pipetting 2ml into two separate conical centrifuge tubes. Use one tube as the "cytotoxicity control" and the other tube "viability control".

4. Add 80µl of Lysis Solution to the cytotoxicity control tube. Add 80µl of water to the viability control tube. Mix both tubes using a vortex mixer on low speed to ensure homogeneity.

5. Add 100µl of fresh culture medium (as a diluent) to each well columns 2–12 of an opaque 96-well plate. Either black plates or white plates may be used. See Section 6 for information about choosing plates.

6. Add 100µl of the cytotoxicity control to each well in columns 1 and 2, rows A, B, C, D. Add 100µl of the viability control to each well in columns 1 and 2, rows E, F, G, H. Starting at column 2, gently mix by aspirating and dispelling 100µl of sample. Repeat three times. Transfer 100µl to column 3, and repeat dilution series through column 11, mixing well after each transfer. Remove and discard 100µl from column 11 after mixing.

Table 1. 96-Well Plate Layout for Determining Linear Range and Sensitivity Method 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
B	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
C	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
D	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
E	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
F	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
G	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells
H	20,000	10,000	5,000	2,500	1,250	625	312	156	78	39	19	no cells

Dying Population: White (rows A–D); Viable Population: Gray (rows E–H)

7. Prepare the CellTox™ Green Reagent (2X) as described in Section 3.A.

8. Add 100µl of the CellTox™ Green Reagent (2X) to all of the wells of the plate prepared in Step 6. Mix briefly by orbital shaking (500–700rpm).

4.A. Determining Linear Range and Sensitivity, Method 1 (continued)

9. Incubate for at least 15 minutes at room temperature (shielded from light) to facilitate dye/DNA binding.

   Notes:

   1. Additional incubation is not detrimental but may not substantially improve the resulting signal.
   2. Depending upon the fluorometer manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900rpm) before measuring fluorescence.

10. Measure fluorescence using an excitation wavelength of 485–500nm and emission of 520–530nm. Adjust the instrument to optimize the dynamic range.

    Note: Avoid signal saturation and machine-limiting signal. Be sure that the signal is within the linear range of the instrument, because signals outside of the linear range will negatively affect the accuracy of the results. Some instrument manufacturers label these points as "over" or "####", indicating saturation. Other instruments insert a maximal default number indicating saturation. Default replicate numbers will be the same value with no variation. Lower the instrument gain if either of these situations occur.

11. Calculate the average fluorescence for the replicates of the nonviable cell populations. Calculate the average fluorescence of the replicates of the viable cell populations. Subtract the average viable cell fluorescence from the average nonviable cell fluorescence. Plot net fluorescence v. cell number, and apply linear curve fit. If the r^2 value is >0.95, then the number of cells used is within the assay's linear range. If the linear fit gives an r^2 value <0.95, omit the data point for the highest number of cells, and recalculate the r^2 value; continue until an r^2 value > 0.95 is achieved. This is the validated linear range of the assay for the cell type.

12. Determine the practical sensitivity for your **cell type** by calculating the signal-to-noise ratio for each dilution of cells (20,000 cells/well; 10,000 cells/well; 5,000 cells/well, etc.).

$$\text{Cytotoxicity S:N} = \frac{\text{(Avg. Fluorescence Value of Nonviable Population} - \text{Avg. Fluorescence Value of Viable Population)}}{\text{Standard Deviation of No-Cell Control}}$$

    Note: The practical level of assay sensitivity for either **assay** is a signal-to-noise ratio of greater than 3 standard deviations (as per the method of Zhang *et al.* 1999; 2).

4.B. Determining Linear Range and Sensitivity, Method 2

Note: This method is written for the Endpoint Method, 2X Reagent Addition, but resulting data are applicable to all assay formats.

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 hemocytometer, then adjust them 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 than 100,000 viable cells/ml. Overall viability of less than 95% will increase background and decrease the assay signal window.

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

4. Create a spectrum of cytotoxicity by blending sonicated (cytotoxicity control) and untreated (viability control) populations in 1.5ml tubes as described in Table 2.

Table 2. Creating a Spectrum of Cytotoxicity and Viability Control Cell Populations.

Percent Cytotoxicity	Volume of Cytotoxicity Control	Volume of Viability Control
100	1,000µl	0µl
98	980µl	20µl
95	950µl	50µl
90	900µl	100µl
75	750µl	250µl
50	500µl	500µl
25	250µl	750µl
10	100µl	900µl
5	50µl	950µl
2	20µl	980µl
0	0µl	1,000µl

5. After mixing each blend of live and dead cells by gently vortexing, pipet 100µl of each blend into 8 replicate wells of a 96-well plate. Add the 100% nonviable cells to column 1, 98% nonviable cells to column 2, etc. Add cell culture medium only to column 12 to serve as the no-cell control.

Table 3. 96-Well Plate Layout for Method 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
B	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
C	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
D	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
E	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
F	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
G	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells
H	100%	98%	95%	90%	75%	50%	25%	10%	5%	2%	0%	no cells

6. Create CellTox™ Green Reagent (2X) as described in Section 3.A.

4.B. Determining Linear Range and Sensitivity, Method 2 (continued)

7. Add 100µl of CellTox™ Green Reagent (2X) to the wells of the plate prepared in Step 5. Mix briefly by orbital shaking (500–700 rpm).

8. Incubate for at least 15 minutes at room temperature (protected from light) to facilitate dye/DNA binding.

   Notes:

   1. Additional incubation is not detrimental but may not substantially improve the resulting signal.
   2. Depending upon the fluorometer manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900rpm) before measuring fluorescence.

9. Measure fluorescence at an excitation wavelength of 485–500nm and an emission of 520–530nm. Adjust the instrument to optimize the dynamic range.

   Note: Avoid signal saturation and machine limiting signal. Be sure that the signal is within the linear range of the instrument because signals outside of the linear range will negatively affect the accuracy of the results. Some instrument manufacturers label these points as "over" or "####", indicating saturation. Other instruments insert a default number indicating saturation. Default replicate numbers will be the same value with no variation. Lower the instrument gain if either of these situations occur.

10. Calculate the average fluorescence for the replicates of the nonviable cell populations. Calculate the average fluorescence of the replicates of the viable cell populations. Subtract the average viable cell fluorescence from the average nonviable cell fluorescence. Plot net fluorescence v. cell number, and apply linear curve fit. If the r^2 value is >0.95, then the number of cells used is within the assay's linear range. If the linear fit gives an r^2 value <0.95, omit the data point for the highest number of cells, and recalculate the r^2 value; continue until an r^2 value > 0.95 is achieved. This is the validated linear range of the assay for the cell type.

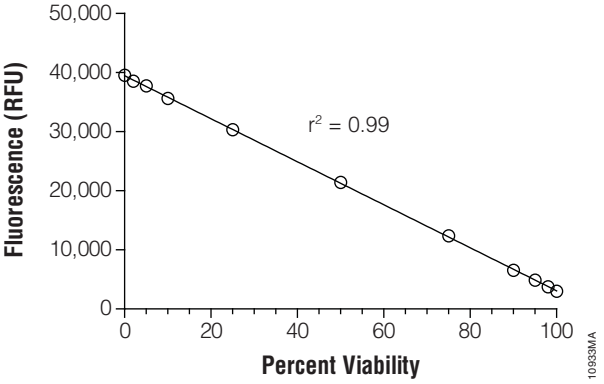

Figure 4. Validated linear range of the assay. Viable and nonviable K562 cells were blended at varying proportions to simulate a nonviable cell spectrum from 0–100% viability. The CellTox™ Green Reagent produces proportional increases in fluorescence with increases in cytotoxicity and can discriminate between 98 and 100% viability in a total population of 10,000 cells/well.

5. User Protocols for the CellTox™ Green Cytotoxicity Assay

Materials to Be Supplied by the User

- opaque-walled 96- or 384-well tissue culture plates compatible with fluorometer (clear- or solid-bottom)
- multichannel pipettor and tips or liquid-dispensing robot
- reagent reservoirs
- multiwell fluorescence plate reader with 485–510nm excitation source and 520–530nm emission filter or monochromator
- vortex and orbital shaker
- cells and medium
- water bath
- hemocytometer and trypan blue

5.A. Endpoint Method, 2X Reagent Addition

1. Adjust cells to the target concentration, and add 50µl of cells to an opaque assay plate (50µl at 100,000–200,000/ml = 5,000–10,000 cells/well in a 96-well format). Include wells without test compound for cytotoxicity and untreated controls and wells without cells for the background control (columns 11 and 12).

   Note: Both black and white plates may be used. Black plates may yield better signal-to-background ratios.

2. Allow adherent cells to attach (unnecessary for suspension cells).

3. Add serially diluted test compound (e.g., 50µl per well in a 96-well format). Include a volume-matched, untreated control (columns 11 and 12).

   Note: An optional control for primary necrosis can be performed: Add Lysis Solution to replicate wells at a ratio of 1:25 (4µl per 100µl of cells) for toxicity control (wells A–D, column 11). This control represents the minimum signal obtainable from proliferating cells at the end of the exposure period, or the maximum dead-cell signal obtainable from nonproliferating cells. The untreated cell population (wells E–H, column 11) will represent the maximal untreated control signal obtainable at the end of an exposure period.

Table 4. 96-Well Plate Layout for Endpoint Protocols.

	1	2	3	4	5	6	7	8	9	10	11	12
A	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
B	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
C	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
D	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
E	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
F	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
G	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
H	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells

cmpd = compound; tox ctrl = toxicity control; untr ctrl = untreated control; wells with no cells are the background control

5.A. Endpoint Method, 2X Reagent Addition (continued)

4. Incubate the plate for the desired exposure period (e.g., 24, 48 or 72 hours).
5. Prepare CellTox™ Green Reagent (2X) as described in Section 3.A., Endpoint Method, 2X Reagent Addition.
6. Add 100µl of the CellTox™ Green Reagent (2X) per well.
7. Mix plate by orbital shaking (700–900rpm) for 1 minute to ensure homogeneity.
8. Incubate at room temperature for 15 minutes, shielded from ambient light.

   Notes:

   1. Additional incubation is not detrimental but may not substantially improve the resulting signal.
   2. Depending upon the fluorometer manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900 rpm) before measuring fluorescence.
9. Measure fluorescence at 485–500nm$_{Ex}$/520–530nm$_{Em}$.

   Note: Avoid signal saturation and machine limiting signal. Be sure that the signal is within the linear range of the instrument because signals outside the linear range will negatively affect the accuracy of the results. Some instrument manufacturers label these points as "over" or "####", indicating saturation. Other instruments insert a default number indicating saturation. Default replicate numbers will be the same value with no variation. Lower the instrument gain if either of these situations occur.

5.B. Endpoint Method, Multiplex (~5X) Reagent Addition

1. Adjust cells to the target concentration and add 50µl of cells to an opaque assay plate (e.g., 50µl at 100,000–200,000/ml = 5,000–10,000 cells/well in a 96-well format). Include wells with no cells for the background control.

   Note: Both black and white plates may be used. Black plates may yield better signal-to-background ratios.
2. Allow adherent cells to attach (unnecessary for suspension cells).
3. Add serially diluted test compound (e.g., 50µl per well in a 96-well format). Include a volume-matched, untreated vehicle control (columns 11 and 12, Table 4).

   Note: An optional control for primary necrosis can be performed: Add Lysis Solution to replicate wells at 1:25 (4µl per 100µl of cells) for toxicity control (wells A–D, column 11, Table 4). This will represent the minimum positive control signal obtainable from proliferating cells at the end of the exposure period, or the maximum dead cell signal obtainable from non-proliferating cells. The untreated cell population (wells E–H, column 11) will represent the maximal negative control signal obtainable at the end of an exposure period.
4. Incubate the cells for the desired exposure period (e.g., 24, 48 or 72 hours).
5. Prepare CellTox™ Green Reagent (~5X) as described in Section 3.B, Reagent Preparation for Endpoint Method, Multiplex Reagent Addition.
6. Add CellTox™ Green Reagent (~5X) to each well to achieve a final concentration of 1X.

7. Mix the plate on an orbital shaker (700–900rpm) for 1 minute to ensure homogeneity.
8. Incubate at room temperature for 15 minutes, shielded from ambient light.

   Notes:

   1. Additional incubation is not detrimental but may not substantially improve the resulting signal.
   2. Depending upon the fluorometer manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900 rpm) before measuring fluorescence.
9. Measure fluorescence intensity at 485–500nm$_{Ex}$/520–530nm$_{Em}$.

   Note: Avoid signal saturation and machine limiting signal, which may artificially impact the linearity of the measurement. Some instrument manufacturers label these points as "over" or "####", indicating saturation. Other instruments insert a default number indicating saturation. Default replicate numbers will be the same value with no variation. Lower the instrument gain if either of these situations occur.
10. Proceed to Section 5.E for sequential multiplexing protocol.

5.C. Express, No-Step Addition at Seeding Method

1. Completely thaw the CellTox™ Green Dye in a 37°C water bath. Mix the CellTox™ Green Dye using a vortex mixer to ensure homogeneity. A brief centrifugation may be necessary to collect the CellTox™ Green Dye in the bottom of the tube.
2. Harvest and adjust the cells to the desired density (e.g., 100,000–200,000 cells/ml) with fresh cell culture medium. Transfer 10µl of the CellTox™ Green Dye to each 5ml of cells. Mix by inversion or gently vortex to ensure dye homogeneity.
3. Pipet the CellTox™ Green Dye/cell suspension to a sterile multiwell plate (50µl per well for 96-well formats, or 12µl per well for 384-well formats).

   Note: Both black and white plates may be used. Black plates may yield better signal-to-background ratios.
4. Allow adherent cells to attach (unnecessary for suspension cells).
5. Add serially diluted test compound (50µl per well for 96-well formats or 12µl per well for 384-well formats). Include a volume-matched, untreated vehicle control (columns 11 and 12).

   Notes:

   1. An optional control for primary necrosis can be performed: Add Lysis Solution to replicate wells at 1:25 (4µl per 100µl of cells) for toxicity control (wells A–D, column 11). This will represent the minimum positive control signal obtainable from proliferating cells at the end of the exposure period or the maximum dead cell signal obtainable from nonproliferating cells. The untreated cell population (wells E–H column 11) will represent the maximal negative control signal obtainable at the end of an exposure period.
   2. Depending upon instrument manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900rpm) before measuring fluorescence.

5.C. Express, No-Step Addition at Seeding Method (continued)

Table 5. 96-Well Plate Layout for Express, No-Step Addition at Cell Seeding Method.

	1	2	3	4	5	6	7	8	9	10	11	12
A	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
B	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
C	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
D	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
E	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
F	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
G	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
H	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells

cmpd = compound; tox ctrl = toxicity control; untr ctrl = untreated control; wells with no cells are the background control wells

6. Measure fluorescence at any point between 0 and 72 hours. Return the plate to the incubator between reads.
7. Proceed to Section 5.E for sequential multiplexing protocol.

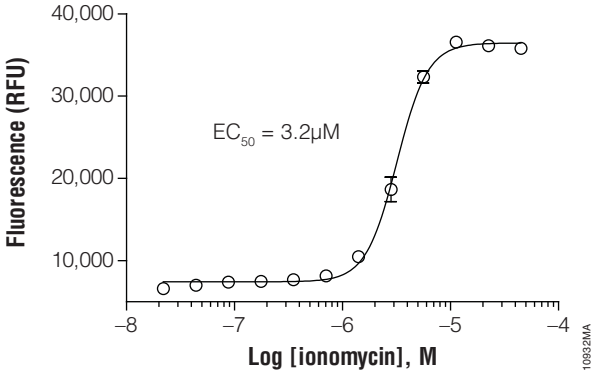

Figure 5. A typical dose-response curve. Ionomycin was applied to K562 cells following the Express, No-Step Addition at Seeding method. Fluorescence was measured after 4 hours of cell exposure to the ionomycin.

5.D. Express, No-Step Addition at Dosing Method (example protocol)

1. Completely thaw the CellTox™ Green Dye in a 37°C water bath. Mix the CellTox™ Green Dye using a vortex mixer to ensure homogeneity. A brief centrifugation may be necessary for complete recovery of the CellTox™ Green Dye.
2. Transfer 10µl CellTox™ Green Dye to each 5ml of compound diluent. Mix the solution using a vortex mixer to ensure homogeneity.

   Note: Prepare enough of CellTox™ Green Dye/diluent to create the initial compound dilution and subsequent serial dilutions.
3. Pipet the CellTox™ Green Dye/diluent into the wells of a sterile multiwell plate and perform serial dilutions of compound (50µl per well for 96-well formats or 12µl per well for 384-well formats).
4. Transfer the cell suspension into the wells containing the serially diluted compounds (50µl per well for 96-well formats, or 12µl per well for 384-well formats).

   Notes:

   1. An optional control for primary necrosis can be performed: Add Lysis Solution to replicate wells at 1:25 (4µl per 100µl of cells) for toxicity control (wells A–D, column 11, Table 6). This will represent the minimum positive control signal obtainable from proliferating cells at the end of the exposure period, or the maximum dead-cell signal obtainable from non-proliferating cells. The untreated cell population (wells E–H, column 11) will represent the maximal negative control signal obtainable at the end of an exposure period.
   2. Depending upon instrument manufacturer and optical detection configuration, replicate wells may benefit from 1 minute of shaking on an orbital shaker (700–900RPM) before measuring fluorescence.

Table 6. 96-Well Plate Layout for Express, No-Step Addition at Dosing Method.

	1	2	3	4	5	6	7	8	9	10	11	12
A	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
B	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
C	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
D	cmpd 1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	tox ctrl	no cells
E	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
F	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
G	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells
H	cmpd 2	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	untr ctrl	no cells

cmpd = compound; tox ctrl = toxicity control; untr ctrl = untreated control; wells with no cells are the background control wells

5. Measure fluorescence at any point between 0 and 72 hours. Return the plate to the incubator between reads.
6. Proceed to Section 5.E for sequential multiplexing protocol.

5.D. Express, No-Step Addition at Dosing Method (example protocol, continued)

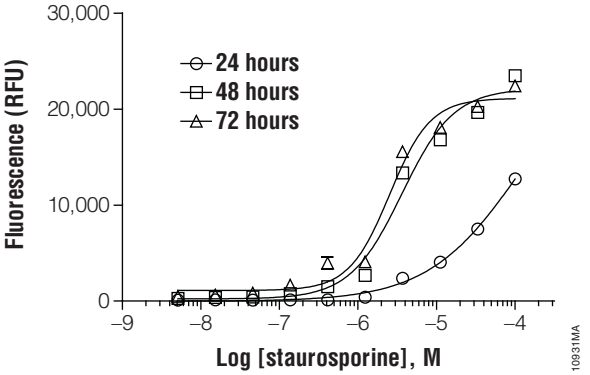

Figure 6. Cytotoxicity is concentration- and exposure- period dependent. Staurosporine was applied to K562 cells after introducing the CellTox™ Green Dye following the Express, No-Step Addition at Seeding method. Fluorescence associated was measured after 24, 48 and 72 hours of cell exposure to monitor the progression of cytotoxicity.

5.E. Basic, Sequential Multiplexing Protocol (with CellTiter-Glo® Assay)

Note: Numerous complementary or orthogonal cell health assay chemistries can be multiplexed with the CellTox™ Green Cytotoxicity Assay to obtain more informative data per well. In most cases, once the CellTox™ Green Cytotoxicity Assay is complete, these chemistries can be applied using the equal-addition protocol, where an equal volume of reagent is added to each well of cells. This protocol uses the CellTiter-Glo® Luminescent Cell Viability Assay as an example of how additional multiplexed chemistries can be applied. A partial list of cell health-related multiplex options is described in Section 6, General Considerations.

1. After the final CellTox™ Green Assay fluorescence measurement, equilibrate the plate to room temperature.
2. Thaw the CellTiter-Glo® Buffer by immersion in a 37°C water bath. Remove the CellTiter-Glo® Buffer when thawed, and equilibrate it to room temperature.
3. Add the contents of the CellTiter-Glo® Buffer to the CellTiter-Glo® Substrate bottle to form the CellTiter-Glo® Reagent. Mix by inversion or using a vortex mixer to ensure homogeneity.
4. Add the CellTiter-Glo® Reagent to the plate containing CellTox™ Green Dye and experimentally treated cells (100µl/well for 96-well formats and 25µl/well for 384-well formats).
5. Place the plate on an orbital shaker, and shake at 500–700rpm to facilitate cell lysis and ATP extraction from the cells.
6. Measure luminescence after 5–10 minutes.

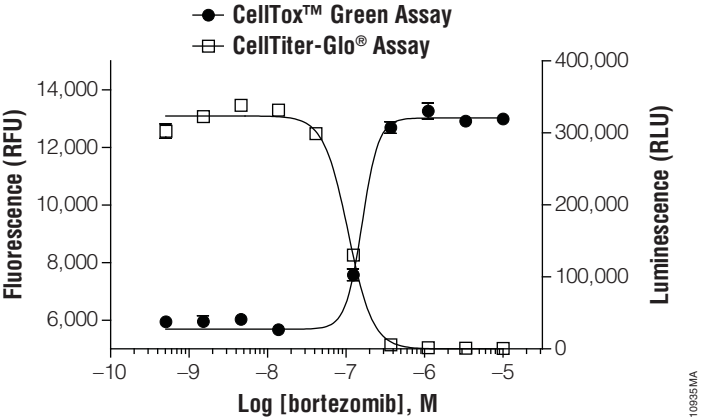

Figure 7. Multiplexing allows complementary measures of cell health. CellTox™ Green Reagent was applied to bortezomib-treated K562 cells after 48 hours of exposure. Fluorescence associated with cytotoxicity was measured, then CellTiter-Glo® Reagent was added and luminescence associated with viability was measured. These inverse measures produce similar EC_{50} values.

6. General Considerations

Assay Interferences

The CellTox™ Green Cytotoxicity Assay is not affected by standard enzymatic interferences resident in other cytotoxicity assays because the CellTox™ Green Dye chemically interacts with DNA exposed as a result of loss of membrane integrity. However, DNA intercalating anti-cancer compounds (doxorubicin, actinomycin D, daunorubicin, etc.) may compete for the same DNA-binding sites, thus reducing CellTox™ Green staining, leading to underestimation of actual cytotoxicity.

The assay is subject to standard fluorescence interferences such as test compound autofluorescence or color quenching. Although statistically rare within the CellTox™ Green Dye excitation and emission spectra, autofluorescent interferences can be revealed by comparing data collected after compound addition (and prior to incubation) to the final endpoint measure. Alternatively, quenching interferences can be discovered by adding compound or vehicle and CellTox™ Green Dye to untreated or Lysis Solution-treated cells and comparing their relative fluorescence values.

Fluorescence Measurements

The CellTox™ Green Dye is optimally excited at 512nm with a peak emission at 532nm. The Blue filter should be used for measurements on the GloMax® Multi+ Instrument. For filter-based instruments, fluorescence should be measured using FITC or rhodamine 110 filters in the range of 485 ± 20nm and 520 ± 20nm. Optimal excitation and emission parameters can be used for monochromator-based instruments, but optical splits should be empirically determined.

6. General Considerations (continued)

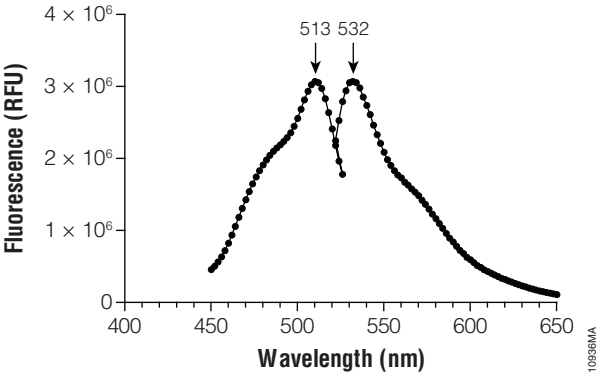

Figure 8. Spectrum of the CellTox™ Green Dye, showing peak excitation and emission.

Fluorescence values can be influenced by well volume. Always normalize well volumes.

Extended incubations (more than 24 hours) can cause evaporation of culture medium (even in humidified incubators), leading to volume gradients from the outer to inner wells of a plate. This can be mitigated by filling the outer wells with up to 300µl (96-well format) of culture medium (and not using these outer wells for anything other than humidification).

Culture System Optimization

Cytotoxicity may be caused by culture conditions (independent of test compound effects) in periods longer than 24 hours due to the accumulation of metabolic waste, pH changes and depletion of nutrients. The CellTox™ Green Dye will be able to access and bind to the DNA of cells with impaired membrane integrity due to culture conditions. Therefore, make every effort to optimize culture conditions and volumes to ensure observed cytotoxicity is compound-dependent. Similarly, use of cell cultures with poor initial viability will substantially increase initial background (untreated signals). Every effort should be made to deliver the same number of viable cells to each assay well.

Serum in Culture Medium

Serum is typically necessary to support cell health and vitality in culture. High concentrations of serum (>20%) may reduce the absolute fluorescence dynamic range (difference between negative and positive control values) of the CellTox™ Green Cytotoxicity Assay but should not adversely affect the signal-to-noise ratio. In general, 5–10% serum is sufficient to support cell health and does not significantly affect assay performance.

Phenol Red and Reagent Delivery

Most commercial formulations of culture medium contain phenol red as a pH indicator. The intrinsic color of phenol red in culture medium can quench overall fluorescence. Each medium formulation will present a different quenching profile that should be explored during the validation stage of CellTox™ Green Cytotoxicity Assay implementation. Endpoint reagent delivery using the provided Assay Buffer can reduce quenching and increase the signal-to-background ratio. The Assay Buffer should not be used to deliver the CellTox™ Green Dye for Express No-Step Addition kinetic formats.

White versus Black Plates

Both white and black plates can be used with the CellTox™ Green Cytotoxicity Assay. However, assay performance (signal-to-background ratio) is typically 2 to 25-fold better in black plates due to higher autofluorescence of white plates. Therefore, use of black plates may be warranted for high-density formats (384- or 1536-well) where fewer cells per well are used. Assay performance is optimal in black plates if performing only the CellTox™ Green Cytotoxicity Assay. White plates are optimal for sequential multiplexing formats that include a luminescent measurement.

Cell Health Multiplex Options

CellTox™ Green Assay can be conveniently multiplexed with many sequential combinations of spectrally distinct chemistries to measure viability or mechanism of toxicity.

First Assay	Second Assay
CellTox™ Green Cytotoxicity Assay	CellTiter-Blue® Assay (viability)
	CellTiter-Fluor™ Assay (viability)
	CellTiter-Glo® Assay (viability)
	GSH/GSSG-Glo™ Assay (mechanism of toxicity)
	Caspase-Glo® 3/7 Assay (mechanism of toxicity)
	P450-Glo™ Assay (mechanism of toxicity)

7. References

1. McDougall, M. and Dwight, S. Nucleic acid binding dyes and uses therefore US Patent Application 2010/0233710 A.
2. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4, 67–73.

8. Related Products

Multiplex Viability and Cytotoxicity Assays

Product	Size	Cat.#
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200

Mechanism-Based Viability and Cytotoxicity Assays

Product	Size	Cat.#
ApoTox-Glo™ Triplex Assay	10ml	G6320
ApoLive-Glo™ Multiplex Assay	10ml	G6410

Viability Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter-Blue® Cell Viability Assay	20ml	G8080

Cytotoxicity Assays

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 2 Assay	10ml	G0940
Caspase-Glo® 3/7 Assay	10ml	G8091
Caspase-Glo® 6 Assay	10ml	G0970
Caspase-Glo® 8 Assay	10ml	G8201
Caspase-Glo® 9 Assay	10ml	G8211
Apo-ONE® Homogeneous Caspase-3/7 Assay	10ml	G7790

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611

Cytochrome P450 Assays

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	10ml	V9001
P450-Glo™ CYP2C9 Assay	10ml	V8791
P450-Glo™ CYP3A4 Assay (Luciferin-PFBE) Cell-Based/Biochemical Assay	10ml	V8901

Detection Instrumentation

Product	Size	Cat.#
GloMax®-Multi+ Detection System with Instinct™ Software:		
Base Instrument with Shaking	each	E8031

[a]Patent Pending.