TECHNICAL BULLETIN

CytoTox-ONE™ Homogeneous Membrane Integrity Assay

Instructions for Use of Products
G7890, G7891 and G7892

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CytoTox-ONE™ Homogeneous Membrane Integrity Assay

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1. Description

The CytoTox-ONE™ Homogeneous Membrane Integrity Assay[a] is a homogeneous, fluorometric method for estimating the number of non-viable cells present in multiwell plates. Cell viability is most often defined based on the integrity of the cell membrane and is most commonly measured by observing the exclusion of trypan blue or other vital dyes. Measurement of the leakage of components from the cytoplasm into the surrounding culture medium has been widely accepted as a valid method to estimate the number of non-viable cells.

The CytoTox-ONE™ Assay is a rapid, fluorescent measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin as shown in Figure 1. The CytoTox-ONE™ Reagent mix does not damage healthy cells; therefore the reactions to measure released LDH can be performed directly in a homogeneous format in assay wells containing a mixed population of viable and damaged cells.

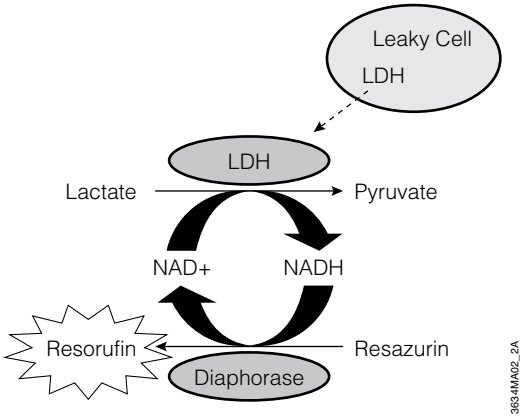

Figure 1. Release of LDH from damaged cells is measured by supplying lactate, NAD+, and resazurin as substrates in the presence of diaphorase. Generation of the fluorescent resorufin product is proportional to the amount of LDH.

The product is supplied as a lyophilized Substrate Mix that is reconstituted with Assay Buffer to form the CytoTox-ONE™ Reagent. A flow diagram summarizing the assay protocol is shown in Figure 2. Assay plates are allowed to equilibrate to ambient temperature, and CytoTox-ONE™ Reagent is added to each well and incubated for 10 minutes. Stop Solution is added, and the fluorescent signal is measured. Fluorescence data are collected using a standard 96- or 384-well fluorometer. The amount of fluorescence produced is proportional to the number of lysed cells using a 96- or 384-well format (Figure 3).

Methods for determining LDH release in conjunction with diaphorase have been used for several years. Variations on this technology have been reported for measuring natural cytotoxicity and have been demonstrated to be identical (within experimental error) to values determined in parallel ^{51}Cr release assays (1,2).

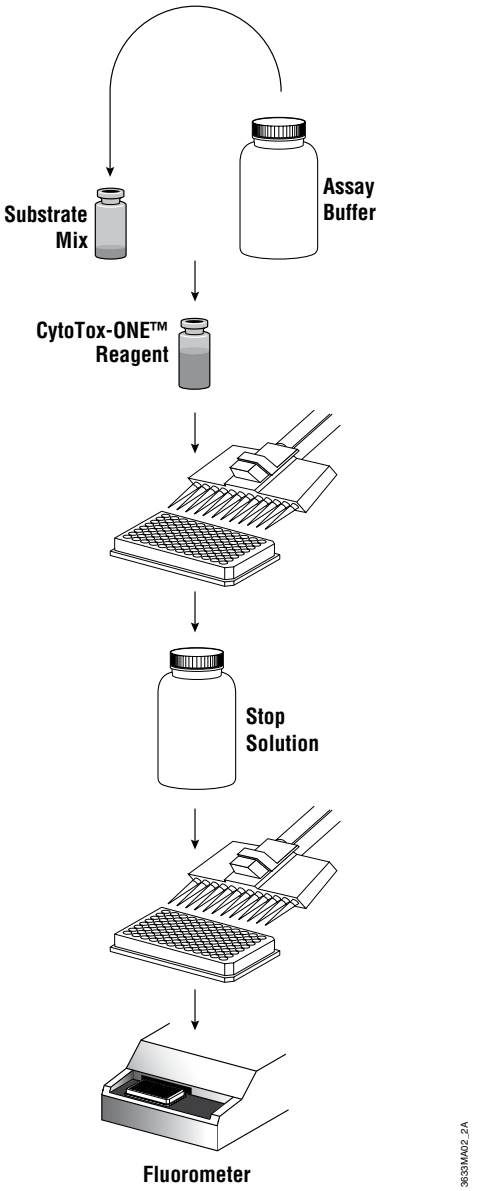

Figure 2. The CytoTox-ONE™ Membrane Integrity Assay protocol. Assay plates are allowed to equilibrate to ambient temperature, and CytoTox-ONE™ Reagent is added to each well and incubated for 10 minutes. Stop Solution is added, and the fluorescent signal is measured.

1. Description (continued)

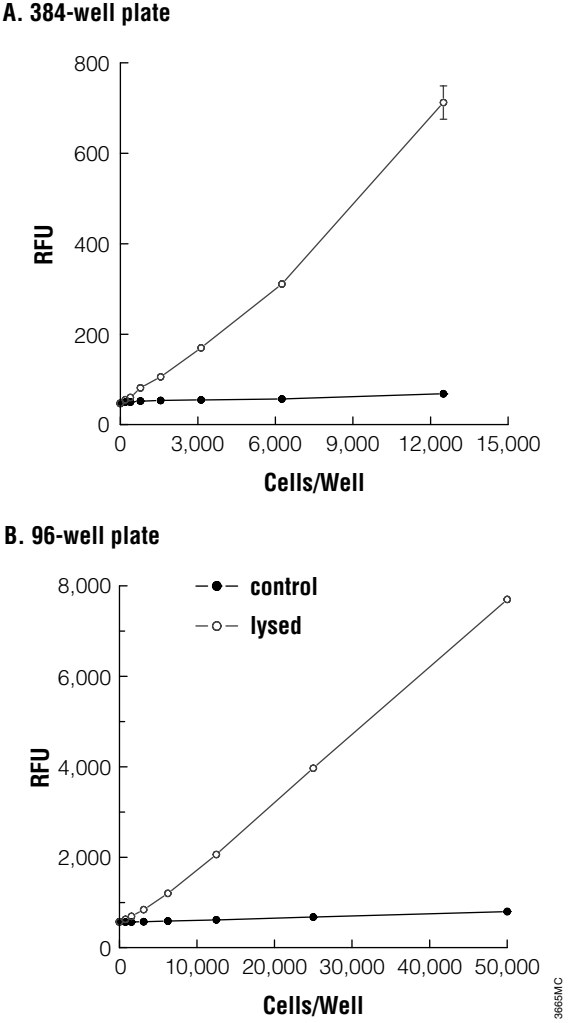

Figure 3. Linear relationship ($r^2 > 0.95$) between cell number and fluorescence ($560_{Ex}/590_{Em}$) using the CytoTox-ONE™ Assay in 384- and 96-well plate formats. Serial twofold dilutions of L929 cells were made in a 384-well plate containing 25µl medium (**Panel A**) and a 96-well plate containing 100µl medium (**Panel B**). Wells were treated by addition of Triton® X-100 to produce "lysed" cells. Wells containing control cells received PBS as the vehicle control. CytoTox-ONE™ Reagent was added following treatments. Values represent the mean ± SD from 4 replicate wells for each cell number. The lowest values shown for each plate format (195 cells for 384-well format and 781 cells for the 96-well format) are significantly different than the "zero" cell background fluorescence.

The LDH release assay is commonly used for testing cytotoxicity of various experimental compounds. Figure 4 shows data generated using the CytoTox-ONE™ Assay and depicts the cytotoxic effect of TNFα on murine L929 cells. Increasing concentrations of TNFα are toxic to L929 cells, resulting in a loss of membrane integrity, release of LDH into the surrounding medium, and a greater fluorescent signal. Figure 4 also shows the results obtained using the CellTiter-Glo® Luminescent Cell Viability Assay to measure ATP content, which estimates the number of viable cells (3,4). Concentrations of TNFα that are toxic to L929 cells result in a loss of ATP and a reduced luminescent signal. The IC_{50} values determined using both assays are similar.

The CytoTox-ONE™ Assay can also be used to determine the total number of cells present in wells at the end of a proliferation assay by adding a Lysis Solution to all wells (5).

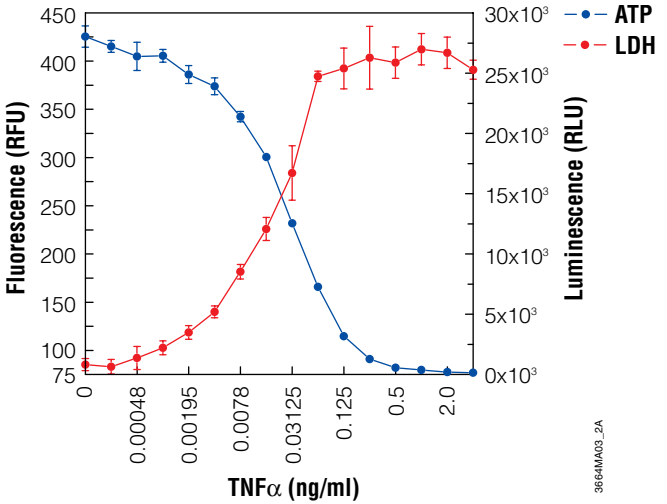

Figure 4. Half-maximal response variables correlate well between the CytoTox™-ONE Assay and other assays. Murine L929 cells were seeded at 2,000 cells per well in a 384-well plate in serum-supplemented medium, cultured for 24 hours, then various amounts of TNFα were added and incubated overnight. Either CytoTox-ONE™ Reagent or CellTiter-Glo® Reagent was added to parallel sets of wells, and fluorescence ($560_{Ex}/590_{Em}$) or luminescence values, respectively, were recorded. (The CellTiter-Glo® Assay uses ATP content to indicate the number of viable cells.) The values represent the mean ± SD of 4 replicate samples. The half maximal response values correlate well for the cytotoxicity and viability assays.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	**200–800 assays**	**G7890**

Each vial contains sufficient reagents to perform 100 assays in a 96-well format or 400 assays in a 384-well format when the recommended volumes are used. Includes:

- 2 vials Substrate Mix
- 24ml Assay Buffer
- 0.5ml Lysis Solution
- 11ml Stop Solution

PRODUCT	SIZE	CAT.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	**1,000–4,000 assays**	**G7891**

Each vial contains sufficient reagents to perform 100 assays in a 96-well format or 400 assays in 384-well format when the recommended volumes are used. Includes:

- 10 vials Substrate Mix
- 120ml Assay Buffer
- 0.5ml Lysis Solution
- 60ml Stop Solution

PRODUCT	SIZE	CAT.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP	**1,000–4,000 assays**	**G7892**

Each vial contains sufficient reagents to perform 500 assays in a 96-well format or 2,000 assays in a 384-well format when the recommended volumes are used. Includes:

- 2 vials Substrate Mix
- 120ml Assay Buffer
- 0.5ml Lysis Solution
- 60ml Stop Solution

Storage Conditions: Store all components frozen at –20°C protected from light. Reconstituted CytoTox-ONE™ Reagent may be stored without loss of activity for up to 3 days at room temperature (22–25°C), up to 1 week at 4°C, or 6–8 weeks at –20°C, protected from light.

3. General Considerations

3.A. Background Fluorescence/Serum LDH

Animal serum used to supplement tissue culture medium may contain significant amounts of LDH that can lead to background fluorescence. The quantity of LDH in animal sera will vary depending on several factors, including the species and the health or treatment of the animal prior to collecting serum. Background fluorescence can be corrected by including a control to measure the fluorescence from serum-supplemented culture medium in the absence of cells. The fluorescence value determined from this control is used to normalize the fluorescence values obtained from other samples. Using reduced serum concentrations or serum-free medium can reduce or eliminate background fluorescence resulting from LDH in serum and improve assay sensitivity.

3.B. Temperature

The generation of fluorescent product in the CytoTox-ONE™ Assay is proportional to the quantity of LDH. The enzymatic activity of LDH is influenced by temperature. We recommend equilibrating the temperature of the assay plate and the CytoTox-ONE™ Reagent to 22°C (20–30 minutes) prior to adding the CytoTox-ONE™ Reagent to initiate the reaction.

The recommended incubation period for the CytoTox-ONE™ Reagent is 10 minutes when reagents and samples are at 22°C. At longer incubation times or higher temperatures, assay linearity may decrease due to substrate depletion. In some situations, the time required for manual or robotic addition of CytoTox-ONE™ Reagent to the assay plate may be a significant portion of the 10-minute incubation period. To minimize the difference in incubation interval among wells within a plate, we recommend adding Stop Solution using the same sequence used for adding the CytoTox-ONE™ Reagent.

3.C. Assay Controls

In a standard cytotoxicity assay, it may be desirable to perform a 100% cell lysis control to determine the maximum amount of LDH present. Individual laboratories may prefer to use a positive control that is known to be toxic for their specific conditions, depending on the cell type, culture conditions, and assay model system. For convenience, we include the Lysis Solution, which is a 9% (weight/volume) solution of Triton® X-100 in water. Use of Lysis Solution at the recommended dilution will result in almost immediate lysis of most cell types and subsequent release of cytoplasmic LDH into the surrounding culture medium. Use of Lysis Solution at the recommended dilution is compatible with the CytoTox-ONE™ Assay chemistry.

3.D. Considerations for the Maximum LDH Release Control

Experimental design will influence the values for the Maximum LDH Release control. The mechanism of cytotoxicity, and thus the kinetics of release of LDH, may vary widely for different experimental compounds being tested. The method by which the Maximum LDH Release Control is prepared as well as the timing of the addition of Lysis Solution (i.e., beginning, middle, or end of experimental/drug treatment period) may both affect the value obtained for 100% LDH release. For example, if the indicator cells are growing throughout the duration of exposure to test compounds, untreated control wells may have more cells and thus may have more LDH present at the end of the exposure period. Adding Lysis Solution after cultured cells are exposed to test compounds may give a different Maximum LDH Release Control value than adding Lysis Solution prior to exposure period.

3.D. Considerations for the Maximum LDH Release Control (continued)

The half-life of LDH that has been released from cells into the surrounding medium is approximately 9 hours. If Lysis Solution is added at the beginning of an experimental exposure period, the quantity of active LDH remaining in the culture medium at the end of the experiment may underestimate the quantity of LDH present in untreated cells.

The recommended dilution of Lysis Solution is compatible with the enzymatic reactions and fluorescence of the assay. Using higher concentrations of Lysis Solution may increase the rate of enzymatic reactions and inflate maximum cell lysis values.

3.E. Light Sensitivity of Resazurin

The resazurin dye in the CytoTox-ONE™ Reagent and the resorufin product formed during the assay are light-sensitive. Prolonged exposure of the Assay Buffer or reconstituted CytoTox-ONE™ Reagent to light will result in increased background fluorescence in the assay and decreased sensitivity.

3.F. Use of Stop Solution to Stop Development of Fluorescent Signal

The Stop Solution provided is designed to rapidly stop the continued generation of fluorescent product and allow the plate to be read at a later time. There may be situations where the researcher will want to take multiple kinetic reads of the same plate and not stop the assay. After adding the Stop Solution, provided that there is some serum (5–10%) present in the samples, the resulting fluorescence is generally stable for up to two days if the assay plate has been protected from light exposure and the wells have been sealed with a plate sealer to prevent evaporation. If no serum is present, the resulting fluorescence is stable for 1–2 hours.

3.G. Cell Culture Media Considerations

Pyruvate-supplemented medium is recommended for some cell lines. Common examples of culture media that contain pyruvate include: Ham's F12, Iscove's, and some formulations of DMEM. Culture media containing pyruvate may cause a reduction in the fluorescent signal due to product inhibition of the LDH reaction catalyzing conversion of lactate to pyruvate. For most situations, the recommended assay conditions of 10 minutes at 22°C will provide adequate signal. However, assay conditions can be empirically optimized.

To increase the fluorescent signal, we recommend determining if the cell line requires pyruvate supplementation during the assay period. Alternatively, conditions known to increase the fluorescent signal include increasing the time of incubation with the CytoTox-ONE™ Reagent prior to adding Stop Solution or incubating the assay at temperatures above the recommended 22°C (up to 37°C). In all cases, all samples should be assayed under the same conditions.

3.H. Use of Resazurin as an Indicator in both Cytotoxicity and Cell Viability Assays

Resazurin reduction is a common reporter for both cytotoxicity and cell viability assays. Using the reaction conditions recommended for the CytoTox-ONE™ Assay (i.e., reduced temperature and short incubation time), only a negligible amount of resazurin is reduced by the viable cell population. Figure 3 (Section 1) shows only a very slight increase in fluorescence in the control (viable) cells. In the CytoTox-ONE™ Assay, the rate of the LDH reaction is increased by providing excess substrates (pyruvate, NAD+, and diaphorase) so that the reaction proceeds relatively quickly (10 minutes at ambient temperature). By contrast, the CellTiter-Blue® Cell Viability Assay requires longer incubation

times (1–4 hours) and a higher incubation temperature (37°C). Additionally, the concentration of resazurin is different between the two assays.

3.I. Performing Other Multiplexed Assays in Combination with the CytoTox-ONE™ Assay

It is possible to remove a portion of the medium from each well to a separate multiwell plate to measure LDH release using the CytoTox-ONE™ Assay followed by performing a separate assay on the sample remaining in the original well. Some examples of this application include estimating the number of viable cells by measuring the ATP content using CellTiter-Glo® Assay or by measuring tetrazolium reduction using the CellTiter 96® AQ_{ueous} One Solution Assay. Alternatively, the level of apoptosis could be determined by measuring the caspase activity using the Apo-ONE® Homogeneous Caspase-3/7 Assay.

4. Protocols

Materials to Be Supplied by the User

- 96- or 384-well opaque-walled tissue culture plates compatible with fluorometer (clear or solid bottom)
- multichannel pipettor
- reservoirs to hold CytoTox-ONE™ Reagent and Stop Solution
- fluorescence plate reader with excitation 530–570nm and emission 580–620nm
- plate shaker

4.A. Reagent Preparation

Equilibrate Substrate Mix and Assay Buffer to 22°C. A 37°C water bath may be used to thaw the Assay Buffer, but the Assay Buffer should not be left at 37°C longer than necessary. **For Cat.# G7890 and G7891**, prepare CytoTox-ONE™ Reagent by adding 11ml of Assay Buffer to each vial of Substrate Mix. Gently mix to dissolve the substrate.

For Cat.# G7892, add 55ml of Assay Buffer to each vial of Substrate Mix. Gently mix to dissolve the substrate.

Protect the reagent from direct light. Unused portions of the CytoTox-ONE™ Reagent may be stored tightly capped at –20°C for 6–8 weeks.

4.B. Recommended Controls

Each of these controls should be performed on each plate being assayed.

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

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.

Maximum LDH Release Control: Set up triplicate wells to determine the Maximum LDH Release Control. Add 2µl of Lysis Solution to the positive control wells before addition of reagent. See Considerations for the Maximum LDH Release Control (Section 3.D) concerning timing of addition.

4.C. Example Cytotoxicity Assay Protocol

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

   Note: For 384-well plates, the recommended culture volume is 25µl per well.

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 desired test exposure period.

4. Remove assay plates from 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).

   Note: If samples cannot be equilibrated to 22°C, reduce incubation time to 5 minutes.

5. **Optional:** If Lysis Solution is used to generate a Maximum LDH Release Control, add 2µl of Lysis Solution (per 100µl original volume) to the positive control wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.

6. Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).

7. Incubate at 22°C for 10 minutes.

8. Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl of Stop Solution. Although this step is optional, it is recommended for consistency.

   Note: We recommend adding the Stop Solution to the wells using the same order of addition that was used for adding the CytoTox-ONE™ Reagent. This is especially important if manual addition of reagent takes a significant amount of time.

9. Shake plate for 10 seconds and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm.

   Note: If the plate is not protected from light, the data should be recorded within one hour to avoid increased background fluorescence.

4.D. Calculation of Results

1. Subtract the average fluorescence values of the culture medium background from all fluorescence values of experimental wells.

2. Use the average fluorescence values from experimental, maximum LDH release, and culture medium background to calculate the percent cytotoxicity for a given experimental treatment.

$$\text{Percent Cytotoxicity} = 100 \times \frac{(\text{Experimental} - \text{Culture Medium Background})}{(\text{Maximum LDH Release} - \text{Culture Medium Background})}$$

4.E. Proliferation Assay Protocol

The CytoTox-ONE™ Assay can be used to estimate the total number of cells in assay wells at the end of a proliferation assay. The procedure involves lysing all the cells to release LDH followed by adding the CytoTox-ONE™ Reagent. The total number of cells present will be directly proportional to the background-subtracted fluorescence values, which represent LDH activity.

1. Set up 96-well assay plates containing cells in culture medium.
2. Add test compounds and vehicle controls to appropriate wells so the final volume is 100µl in each well (25µl per well for 384-well plates).
3. Culture cells for desired test exposure period.
4. Add 2µl of Lysis Solution (per 100µl of original volume) to all wells. If a larger pipetting volume is desired, use 10µl of a 1:5 dilution of Lysis Solution.
5. Remove assay plates from 37°C incubator and equilibrate to 22°C (approximately 20–30 minutes).

   Note: If samples cannot be equilibrated to 22°C, reduce incubation time to 5 minutes.
6. Add a volume of CytoTox-ONE™ Reagent equal to the volume of cell culture medium present in each well and mix or shake for 30 seconds (e.g., add 100µl of CytoTox-ONE™ Reagent to 100µl of medium containing cells for the 96-well plate format or add 25µl of CytoTox-ONE™ Reagent to 25µl of medium containing cells for the 384-well format).
7. Incubate at 22°C for 10 minutes.
8. Add 50µl of Stop Solution (per 100µl of CytoTox-ONE™ Reagent added) to each well in the 96-well format. For the 384-well format (where 25µl of CytoTox-ONE™ Reagent was added), add 12.5µl Stop Solution. Although this step is optional, it is recommended for consistency.

   Note: We recommend adding the Stop Solution to the wells using the same order of addition that was used for adding the CytoTox-ONE™ Reagent. This is especially important if manual addition of reagent takes a significant amount of time.
9. Shake plate 10 seconds and record fluorescence 560nm/590nm.

   Note: If the plate is not protected from light, the data should be recorded within one hour to avoid increased background fluorescence.

5. Troubleshooting

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

Symptoms	Causes and Comments
High background	Endogenous LDH in animal sera in culture medium. This background absorbance is normally subtracted as the culture medium background. To reduce the background absorbance, change the source of serum or reduce the serum concentration. LDH activity in sera varies with human AB serum, horse serum, fetal bovine serum and calf serum containing increasing levels of LDH activity. In general, decreasing serum concentration to 5% will significantly reduce background without affecting cell viability.
Low percent cytotoxicity	Percent cytotoxicity too low for convenient quantitation. In cases where it is desirable to increase percent cytotoxicity, increase the incubation time with cytotoxic compounds.
Fluorescence values above linear range of plate reader	Too much LDH activity. Repeat assay and shorten LDH reaction time to 5 minutes. Be sure temperature is equilibrated to 22°C.
Low overall fluorescence	Cell culture medium contains pyruvate. Pyruvate may cause a reduction in the fluorescent signal because of product inhibition of the LDH reaction. Determine if the cell line requires pyruvate supplement during the assay period. Conditions known to increase signal are increasing the incubation time with the CytoTox-ONE™ Reagent and incubating the assay above the recommended 22°C.
	Plate reader set at incorrect gain. Adjust gain setting.
	Improper filter set. Choose appropriate excitation and emission filters. The excitation wavelength should be 560nm, and the emission wavelength should be 590nm.
Unexpected high fluorescence	Experimental compound being tested may be fluorescent. Perform control without addition of CytoTox-ONE™ Reagent to test fluorescence of compound.
	Experimental compound being tested may interfere with assay chemistry. Test combination of experimental compound and CytoTox-ONE™ Reagent without cells.

6. References

1. Korzeniewski, C. and Callewaert, D.M. (1983) An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods* **64**, 313–20.
2. Decker, T. and Lohmann-Matthes, M.L. (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J. Immunol. Methods* **115**, 61–9.
3. *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin* #TB288, Promega Corporation.
4. Crouch, S.P. *et al.* (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **160**, 81–8.
5. Moravec, R. (1994) Total cell quantitation using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. *Promega Notes* **45**, 11–2.

7. Related Products

Product	Size	Cat.#
CytoTox 96® Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780
CellTiter-Glo® Luminescent Cell Viability Assay (ATP, luminescent)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter-Blue® Cell Viability Assay (resazurin, fluorometric)	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS, colorimetric)	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581
CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT, colorimetric)	1,000 assays	G4000
	5,000 assays	G4100

7. Related Products (continued)

Product	Size	Cat.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791
Caspase-Glo® 3/7 Assay (luminescent)	2.5ml	G8090
	10ml	G8091
	10 × 10ml	G8093
	100ml	G8092
Caspase-Glo® 8 Assay (luminescent)	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay (luminescent)	2.5ml	G8210
	10ml	G8211
	100ml	G8212

[a]U.S. Pat. Nos. 6,982,152 B2 has been issued to Promega Corporation for cytotoxicity assay. Other patents are pending.

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