TECHNICAL MANUAL

ROS-Glo™ H_2O_2 Assay

Instructions for Use of Products
G8820 and G8821

Revised 12/23
TM391

ROS-Glo™ H_2O_2 Assay

All technical literature is available at: www.promega.com/protocols/
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1. Description

The ROS-Glo™ H_2O_2 Assay[a] is a homogeneous, rapid and sensitive luminescent assay that measures the level of hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), directly in cell culture or in defined enzyme reactions. This assay allows identification of conditions or test compounds, such as small molecule inhibitors or inducers, that alter ROS levels. The scalable multiwell format couples a stable luminescent signal to the level of H_2O_2 in a sample.

ROS that are generated in cells can act as signaling molecules and, in excess, can lead to cell damage or death (1). The variety of ROS generated in cell cultures or enzyme reactions includes superoxide, hydroxyl radical, singlet oxygen and H_2O_2 (2). H_2O_2 is convenient to assay because it has the longest half-life of all ROS in cultured cells. In addition, various ROS are converted to H_2O_2 within cells (2,3). For example, superoxide dismutase converts superoxide to O_2 and H_2O_2. A change in H_2O_2 can reflect a general change in the ROS level.

The ROS-Glo™ Assay mechanism for H_2O_2 measurement is shown in Figure 1. An H_2O_2 Substrate is employed that reacts directly with H_2O_2 to generate a luciferin precursor. Upon addition of ROS-Glo™ Detection Reagent containing Ultra-Glo™ Recombinant Luciferase and D-Cysteine, the precursor is converted to luciferin by the D-Cysteine, and the produced luciferin reacts with Ultra-Glo™ Recombinant Luciferase to generate a luminescent signal that is proportional to H_2O_2 concentration.

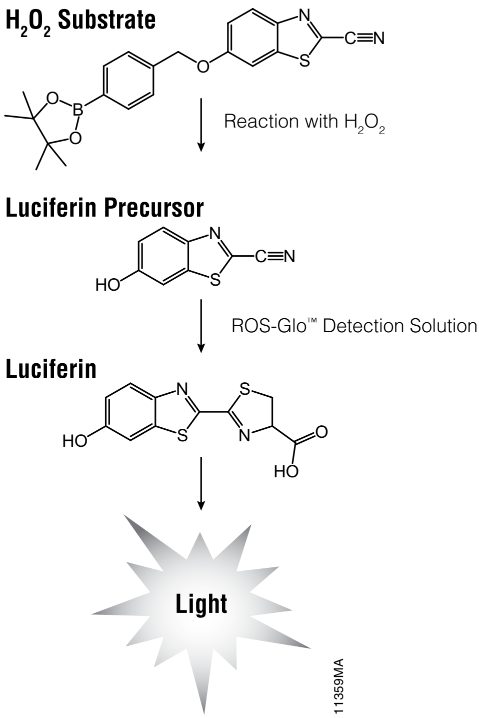

Figure 1. ROS-Glo™ H_2O_2 Assay chemistry.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
ROS-Glo™ H_2O_2 Assay	**10ml**	**G8820**

Cat.# G8820 contains sufficient reagents to perform 100 assays in a 96-well plate using 100µl/well of prepared ROS-Glo™ Detection Solution. Includes:

- 40µl H_2O_2 Substrate, 10mM
- 100µl Signal Enhancer Solution
- 100µl D-Cysteine, 100X
- 2ml H_2O_2 Substrate Dilution Buffer
- 1 vial Luciferin Detection Reagent
- 10ml Reconstitution Buffer

PRODUCT	SIZE	CAT.#
ROS-Glo™ H_2O_2 Assay	**50ml**	**G8821**

Cat.# G8821 contains sufficient reagents to perform 500 assays in a 96-well plate using 100µl/well of prepared ROS-Glo™ Detection Solution. Includes:

- 200µl H_2O_2 Substrate, 10mM
- 500µl Signal Enhancer Solution
- 500µl D-Cysteine, 100X
- 10ml H_2O_2 Substrate Dilution Buffer
- 1 vial Luciferin Detection Reagent
- 50ml Reconstitution Buffer

Storage Conditions: Store all components at –30°C to –10°C. Reconstituted Luciferin Detection Reagent is prepared by adding thawed Reconstitution Buffer to the Luciferin Detection Reagent. When reconstituted, Luciferin Detection Reagent can be stored at room temperature (22–25°C) for 24 hours or at –30°C to –10°C for 3 months with no loss of activity. Best results are obtained by freezing the reconstituted Luciferin Detection Reagent in single-use aliquots.

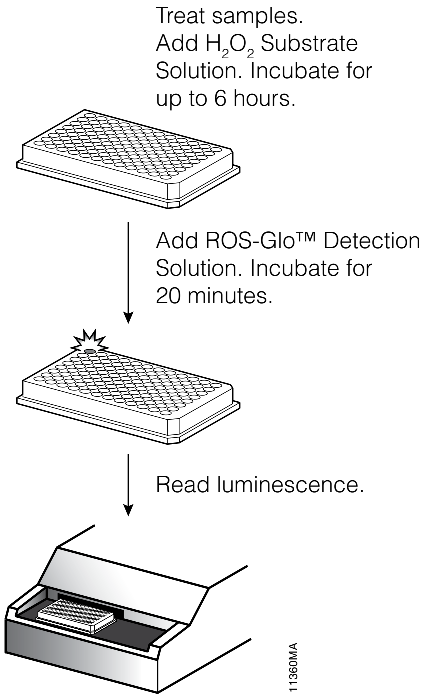

Figure 2. Overview of ROS-Glo™ H_2O_2 Assay protocol for cell-based and biochemical detection.

3. Performing Cell-Based Assays

3.A. General Considerations

The ROS-Glo™ H_2O_2 Substrate can be incubated directly with cells in culture medium or balanced salt solution and the H_2O_2-dependent luminescence read from the same well (Figure 2). The cell-based assay also allows multiplexing with other assays. For example, after performing a nonlytic assay as described in this section, the cells remaining in the initial plate can be assayed for other parameters, such as cell viability or cytotoxicity (see Section 5.A, Cell-Based Assay Multiplexing Protocols).

Materials to Be Supplied by the User

- cell culture medium or balanced salt solution
- opaque, multiwell plates (96-, 384- or 1536-well); white plates or white-walled plates with clear bottoms are recommended. **Note:** White plates are optimal for luminescence; clear bottoms facilitate viewing of cells but may contribute to cross-talk between wells.
- luminometer capable of reading multiwell plates
- **Optional:** multichannel pipette, automated repeating pipettor or liquid-dispensing robot

3.B. Preparation of ROS-Glo™ Detection Solution

1. Transfer the contents of one bottle of thawed Reconstitution Buffer to the amber bottle of lyophilized Luciferin Detection Reagent to produce Reconstituted Luciferin Detection Reagent.

   Note: For storage we recommend freezing the Reconstituted Luciferin Detection Reagent in single-use aliquots.
2. Immediately before use, add 10µl each of D-Cysteine and Signal Enhancer Solution per 1ml of Luciferin Detection Reagent to produce the ROS-Glo™ Detection Solution.
3. Prepare sufficient fresh ROS-Glo™ Detection Solution for the number of samples desired at 100µl per sample in 96-well plates as follows (adjust volumes proportionately for other plate formats and well volumes):

Number of Reaction Wells	Luciferin Detection Reagent	D-Cysteine	Signal Enhancer Solution
10	1ml	10µl	10µl
50	5ml	50µl	50µl
100	10ml	100µl	100µl

3.C. Recommended Samples and Control Reactions

I. Medium without cells, plus the vehicle used for test compounds (e.g., DMSO).

II. Medium with cells, plus the vehicle used for test compounds (e.g., DMSO).

III. Medium without cells, plus test compound.

IV. Medium with cells, plus test compound.

V. Optional positive control H_2O_2 inducer: 50µM menadione in medium, plus and minus cells.

See Figures 3, 4 and 5 for examples of outcomes using these controls.

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3.D. Cell-Based Assay Protocol

The following reagent preparation and volumes are recommended for a cell-based ROS-Glo™ H_2O_2 Assay in 96-well plate format. Volumes can be scaled proportionately for other plate formats and well volumes. Include controls as indicated in Section 3.C.

Homogeneous Assay (Lytic)

1. Add cells at desired density in ≤80µl of medium to 96-well plates (use opaque, white plates or white-walled plates with clear bottoms). Less than 80µl is desirable to accommodate addition of test compounds (e.g., 70µl of cell culture mix plus 10µl of test compound). However, test compounds also may be added at Step 2. with the H_2O_2 Substrate. For adherent cells allow sufficient time for attachment to plate (e.g., 24 hours at 37°C in a CO_2 incubator or an incubation time according to your typical experimental protocol).

   Note: For suspension cell lines, proceed to experimental treatments (Step 3).

   Test compounds such as drugs or other small molecules may be added with the H_2O_2 Substrate Solution.

   Note: We reccomend keeping the final concentration of solvents such as DMSO to ≤1%.

   Add the test compound vehicle to minus-test-compound control samples (e.g., DMSO at the same concentration as the test compound).

2. Thaw the H_2O_2 Substrate Dilution Buffer and place it on ice. Prepare the H_2O_2 Substrate and test compound solution using the chilled H_2O_2 Substrate Dilution Buffer. Dilute the 10mM H_2O_2 Substrate provided in the kit to 125µM in H_2O_2 Substrate Dilution Buffer. If the solution is cloudy, vortex to optimize mixing. Just before use, prepare an amount of H_2O_2 Substrate Solution sufficient for all samples including controls. For a 96-well plate, prepare the following:

Number of Wells	H_2O_2 Substrate Dilution Buffer	H_2O_2 Substrate
10	200µl	2.5µl
50	1.0ml	12.5µl
100	2.0ml	25µl

3. Add 20µl of H_2O_2 Substrate solution (or 20µl of combined H_2O_2 Substrate and test compound) to cells and mix. The final well volume will be 100µl, and the final H_2O_2 Substrate concentration will be 25µM.

4. Place cells in an incubator (e.g., 37°C CO_2 incubator) for the desired treatment time.

   Note: If experimental treatment time is longer than 6 hours, we reccomend adding the H_2O_2 Substrate for the final 6 hours of treatment. For example, if the cells are to be treated with test compound for 24 hours, add the test compound first, incubate the cells for 18 hours, then add the H_2O_2 Substrate Solution and return the plate to the incubator for the final 6 hours of treatment.

3.D. Cell-Based Assay Protocol (continued)

Homogeneous Assay (Lytic; continued)

5. Add 100µl of ROS-Glo™ Detection Solution (prepared in Section 3.B) to each well.
6. Incubate for 20 minutes at room temperature (22–25°C).
7. Record relative luminescence units using a plate reader.

Nonlytic Assay

The nonlytic assay preserves cells for downstream applications. Medium samples are transferred to a separate plate after exposure to the H_2O_2 Substrate (Step 3 of Homogeneous Assay) and combined with an equal volume of ROS-Glo™ Detection Solution.

1. After Step 4 of the Homogeneous Assay Protocol, combine 50µl of medium from each sample well with 50µl of ROS-Glo™ Detection Solution in a separate opaque white plate.
2. Incubate for 20 minutes at room temperature.
3. Record relative luminescence units (RLU) using a plate reader.
4. Cells in the original sample plate can be assayed separately for other parameters, such as cell viability (see Section 5.A, Multiplexing Protocols).

3.E. Data Analysis for Cell-Based ROS-Glo™ H_2O_2 Assays

This section provides a brief explanation of data that may be obtained and how the data are interpreted using the recommended samples and controls described in Section 3.C. Interpretation of ROS measurements from cells in culture takes into account the biochemistry of H_2O_2 production and elimination, and the interplay between these processes and the cell culture medium and/or experimental test compounds. Before examining potential experimental outcomes, note this list of essential aspects of H_2O_2 dynamics in cell culture systems:

- H_2O_2 is cell membrane permeable. When produced inside cells, it diffuses into the medium, and when produced in the medium, it diffuses into cells. ROS-Glo™ H_2O_2 Assay detects H_2O_2 in the well without regard to its source.
- Cultured cells have a strong capacity to eliminate H_2O_2.
- Certain compounds cause cells to produce H_2O_2.
- Certain compounds undergo reactions in cell culture medium that produce H_2O_2 independent of cells (abiotic ROS production).
- Certain cell culture media contain significant amounts of H_2O_2 (likely due to oxidation of medium components), and certain media contain components that react with and eliminate H_2O_2.

To facilitate data analysis, consider the samples and controls (I–V) described in Section 3.C.

3.E. Data Analysis for Cell-Based ROS-Glo™ H_2O_2 Assays (continued)

Potential Outcomes

Test compound produces more H_2O_2 than vehicle alone when applied to cells:

- Control IV (medium with cells plus test compound) > Control II (medium with cells plus vehicle control)

This is a common outcome with compounds such as menadione (control V) that cause oxidative stress and induce ROS production by cells (4; Figure 3).

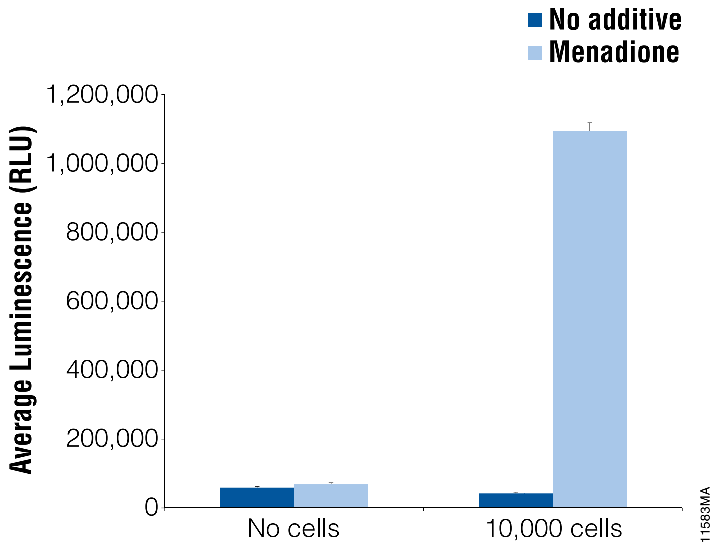

Figure 3. ROS-Glo™ Assay signals from wells +/– menadione and +/– HepG2 cells. HepG2 cells were plated at a density of 10,000 cells/well in a 96-well white cell culture plate, in 100µl of MEM medium with 10% FBS. The same amount (100µl) of MEM with 10% FBS was added to control wells without cells. After overnight incubation at 37°C in 5% CO_2, medium was removed from all wells. Eighty microliters of MEM with 0.5mM pyruvate, and 20µl of H_2O_2 Substrate Dilution Buffer with 125µM H_2O_2 Substrate, with either no menadione or 20µM menadione, was added to wells with and without cells. The plate was returned to the incubator for 2 hours, then 100µl of ROS-Glo™ Detection Solution was added to the wells. The plate was incubated for 20 minutes at room temperature, and the luminescence was determined with a GloMax® Luminometer. The average RLU and standard deviation of quadruplicate samples were calculated.

3.E. Data Analysis for Cell-Based ROS-Glo™ H_2O_2 Assays (continued)

Potential Outcomes

Test compound produces more H_2O_2 in medium without cells than in medium with cells:

- Control III (medium without cells plus test compound) > Control IV (medium with cells plus test compound).

This is a common outcome for compounds such as polyphenols that undergo an abiotic reaction in medium to produce H_2O_2 (5,6). This H_2O_2 diffuses into cells and is actively eliminated (e.g., by way of intracellular catalase activity). The comparison of samples with cells to controls without cells that are otherwise identical enables discrimination between cell-dependent and cell-independent changes in ROS levels (Figure 4).

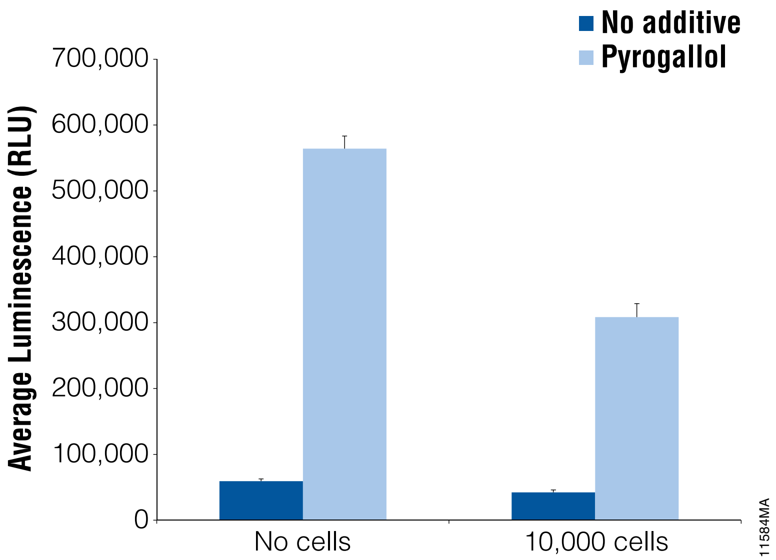

Figure 4. ROS-Glo™ Assay signals from wells +/– pyrogallol and +/– HepG2 cells. HepG2 cells were plated at a density of 10,000 cells/well in a 96-well white cell culture plate in 100µl of MEM medium containing 10% FBS. MEM (100µl) containing 10% FBS was added to control wells without cells. After overnight incubation at 37°C in 5% CO_2, the medium was removed from all wells. Eighty microliters of MEM medium with 0.5mM pyruvate, and 20µl of H_2O_2 Substrate Dilution Buffer containing 125µM H_2O_2 Substrate, with either no pyrogallol or 20µM pyrogallol, was added to cells with and without wells. The plate was returned to the incubator for 2 hours. After incubation, 100µl of ROS-Glo™ Detection Solution was added to the wells. The plate was incubated for 20 minutes at room temperature. Luminescence was determined with a Glo-Max® Luminometer. The average RLU and standard deviation of quadruplicate samples were calculated.

3.E. Data Analysis for Cell-Based ROS-Glo™ H_2O_2 Assays (continued)

Potential Outcomes

Cell-free medium shows stronger signal than medium with cells:

- Control I (medium without cells plus vehicle control) > Control II (medium with cells plus vehicle control).

This is observed with certain media that contain some H_2O_2 (likely produced via spontaneous oxidation of medium components) (7). When applied to cells the H_2O_2 content of such media is decreased by the active capacity of cells to eliminate H_2O_2 (e.g., by way of intracellular catalase activity; see Figure 5).

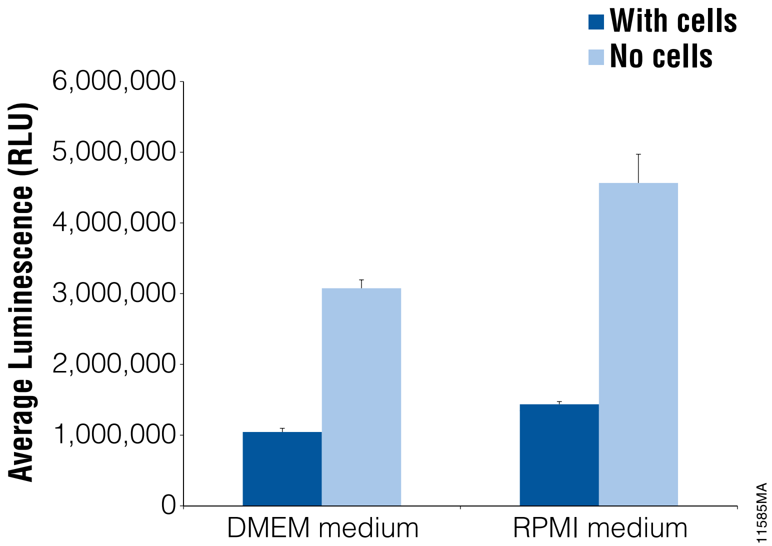

Figure 5. ROS-Glo™ Assay signals from wells with DMEM or RPMI medium +/– HepG2 cells. HepG2 cells were plated at a density of 10,000 cells/well in a 96-well white cell culture plate in 100µl of DMEM medium containing 10% FBS. After overnight incubation at 37°C in 5% CO_2, medium was removed from all wells. Eighty microliters of DMEM medium with 0.5mM pyruvate and 20µl of H_2O_2 Substrate Dilution Buffer containing 125µM Substrate was added to wells with and without cells. Other wells with and without cells were treated with 80µl of RPMI 1640 medium with 0.5mM pyruvate and 20µl of H_2O_2 Substrate Dilution Buffer containing 125µM ROS-Glo™ H_2O_2 Substrate. The plate was returned to the incubator for 2 hours. After incubation, 100µl of ROS-Glo™ Detection Solution was added to the wells. The plate was incubated for 20 minutes at room temperature. Luminescence was determined with a GloMax® Multi+ Luminometer. The average RLU and standard deviation of quadruplicate samples were calculated.

Different Levels of H_2O_2 in Different Cell Culture Media

Some media, particularly those containing pyruvic acid, can abiotically eliminate hydrogen peroxide; others generate H_2O_2 abiotically (7). Thus, different signals may be seen if a compound is tested in a medium that eliminates H_2O_2 versus one that generates H_2O_2 or that makes no contribution to an H_2O_2-dependent signal. See Figure 5.

4. Performing Enzyme Reactions

4.A. General Considerations

The ROS-Glo™ H_2O_2 Assay can be used to measure the production or consumption of H_2O_2 by a purified or partially purified enzyme. The assay chemistry is shown in Figure 1.

Materials To Be Supplied by the User

- enzyme reaction buffer and enzyme of interest
- white, opaque, polystyrene, assay plates (96-, 384- or 1536-well)
- luminometer capable of reading multiwell plates
- 1M Tris-HCl (pH 8.0)
- **optional:** multichannel pipette, automated repeating pipettor or liquid-dispensing robot

4.B. Preparation of ROS-Glo™ Detection Solution

Prepare ROS-Glo™ Detection Solution immediately prior to use.

1. Transfer the contents of one bottle of thawed Reconstitution Buffer to the amber bottle of lyophilized Luciferin Detection Reagent to produce reconstituted Luciferin Detection Reagent.

   Note: We reccomend freezing the reconstituted Luciferin Detection Reagent in single-use aliquots.

2. Just before use, add 10µl each of D-Cysteine and Signal Enhancer Solution per 1ml of Luciferin Detection Reagent to produce the ROS-Glo™ Detection Solution. Prepare a new supply of this solution for each experiment.

   Prepare sufficient ROS-Glo™ Detection Solution for the number of reactions desired for a standard 96-well plate as follows:

Number of Reaction Wells	Luciferin Detection Reagent	D-Cysteine	Signal Enhancer Solution
10	1ml	10µl	10µl
50	5ml	50µl	50µl
100	10ml	100µl	100µl

Volumes can be adjusted proportionately for other plate formats and well volumes.

4.C. Recommended Samples and Control Reactions

Consider the following samples and controls:

I. Measure the enzyme-independent background signal by including samples that lack an essential reaction component (e.g., the enzyme itself or a requisite substrate or cofactor).

II. Compare active enzyme reactions with test compounds such as drugs or other small molecules to active reactions with test compound vehicle only (e.g., DMSO at same concentration as present with test compound).

III. Some small-molecule test compounds generate H_2O_2 in solution in the absence of enzymatic activity (e.g., polyphenols). Control for these by comparing them with test compounds applied to inactive enzyme assay samples. Consider applying this control only with compounds previously identified as H_2O_2 inducers in the enzyme assay.

4.D. Enzyme Assay Protocol

The following reagent preparation and volumes are recommended for use with the ROS-Glo™ H_2O_2 Assay in a 96-well plate format. Volumes can be adjusted proportionately for other plate formats and well volumes. Include controls as indicated in Section 4.C.

Prepare Assay Solutions

The H_2O_2 reaction with the H_2O_2 Substrate is optimal at pH 7.0–9.0. If the enzymatic reaction under investigation is outside of this range, the H_2O_2 Substrate Dilution Buffer can be replaced by 1M Tris-HCl (pH 8.0) to bring the pH to within the pH 7.0–9.0 range.

1. Prepare an Enzyme Reaction Mixture specific for your enzyme of interest at ≤80µl per reaction according to standard guidelines for that enzyme.

2. Prepare a 125µM H_2O_2 Substrate solution by diluting the 10mM H_2O_2 Substrate in H_2O_2 Substrate Dilution Buffer. Vortex the solution, if it appears cloudy, to optimize mixing. Prepare 20µl per reaction as follows:

Number of Wells	H_2O_2 Substrate Dilution Buffer	H_2O_2 Substrate
10	0.2ml	2.5µl
50	1.0ml	12.5µl
100	2.0ml	25µl

3. Prepare sufficient ROS-Glo™ Detection Solution for the number of controls and reactions desired as described in Section 4.C.

Protocol

If the enzyme reaction is run at pH 7.5–9.0 for 6 hours or less, the H_2O_2 Substrate can be added at the start of the enzymatic reaction. For example, the H_2O_2 Substrate can be added at the same time as test compound dosing.

1. Add ≤80µl of Enzyme Reaction Mix per well to a white opaque 96-well plate.

   Note: Less than 80µl may be applied to accommodate an added volume of test compounds (e.g., 70µl of enzyme reaction mix plus 10µl of test compound).

2. Add test compounds (such as drugs or other small molecules or vehicle) to controls without a test compound (e.g., DMSO in same concentration as present in wells with test compounds).

3. Incubate enzyme reaction at desired temperature (e.g., 37°C or room temperature) for the desired length of time.

   Note: Various methods of initiating and terminating enzyme reactions may be employed. For example, reactions may be initiated by adding an essential reaction component and stopped by adding an enzyme inhibitor. The final reaction volume should be 80µl to allow for addition of H_2O_2 Substrate solution.

4. After the enzyme reaction incubation, add 20µl of H_2O_2 Substrate solution and mix.

5. Incubate the reaction for 60 minutes at room temperature (approximately 22°C).

6. Add 100µl of ROS-Glo™ Detection Solution as prepared in Section 4.B.

7. Incubate 20 minutes at room temperature.

8. Read relative luminescent values (RLU) using a plate-reading luminometer.

4.E. Optional Conversion of Relative Luminescence Unit (RLU) Values to H_2O_2 Concentration

Conversion of relative luminescent values to H_2O_2 concentration can be accomplished by comparing RLU values from samples of unknown H_2O_2 concentration to RLU values of samples in an H_2O_2 standard curve as follows:

1. Prepare a dilution series of pure H_2O_2 in enzyme reaction buffer at the same volumes as the test samples. The low and high end of the H_2O_2 dilution series should generate RLU values that bracket the sample RLUs (e.g., 0.01–10µM H_2O_2 for most assays).

2. Follow Steps 4–8 of the Enzyme Assay Protocol (Section 4.D).

3. Use linear regression analysis of the standard curve to interpolate the H_2O_2 concentration of samples.
   Note: Enzyme-independent background values subtracted from sample values are also subtracted from the standards.

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4.F. Data Analysis for ROS-Glo™ Enzyme Activity Assay

This section provides data processing suggestions and short explanations for results that may be obtained.

- Net signal calculations: Subtract enzyme-independent background (control I, Section 4.C) from all enzyme-active reactions, plus or minus test compounds.
- Compounds that nonenzymatically elevate the H_2O_2 level enhance the enzyme-independent background signal (control III, Section 4.C). For this type of compound the nonenzymatic background is measured with test compound present for the same length of time as with the active enzyme reactions.

5. Appendix

5.A. Cell-Based Assay Multiplexing Protocols

Numerous complementary or orthogonal cell health assay chemistries from Promega can be multiplexed with the ROS-Glo™ H_2O_2 Assay to obtain more informative data per well. In most cases, these chemistries can be applied as described in their standard equal addition protocols. This appendix describes two multiplexing protocols: 1) CellTox™ Green Cytotoxicity Assay and 2) CellTiter-Glo® Cell Viability Assay as examples of how additional multiplexed chemistries can be applied.

Multiplex ROS-Glo™ H_2O_2 Assay and CellTox™ Green Cytotoxicity Assay

The CellTox™ Green Cytotoxicity Assay correlates the dead cell content of a sample to a fluorescent signal. This assay is available from Promega (Cat.# G8731, G8741, G8742, G8743).

Note: For the CellTox™ Green Cytotoxicity Assay, follow the Express, No-Step Addition at Dosing Method (Section 5.D of the *CellTox™ Green Cytotoxicity Assay Technical Manual* #TM375).

1. Apply cells to 96-well assay plate at desired density in 40µl of medium in opaque, white plates or white-walled plates with clear bottoms.

   For adherent cells allow sufficient time for attachment to plate (e.g., 24 hours at 37°C in a CO_2 incubator or an incubation time according to your typical experimental protocol).

   For suspension cell lines proceed to experimental treatments.
2. 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.
3. Prepare the CellTox™ Green Dye and test compound solution in medium. Combine 10µl of CellTox™ Green Dye and 5ml of test compound diluent medium. Mix the solution using a vortex mixer to ensure homogeneity.

   Add test compounds such as drugs and other small molecules to samples up to the desired concentration in a vehicle appropriate for the experiment (e.g., DMSO). **Note:** Keep the final concentration of a solvent such as DMSO to ≤1%.

Multiplex ROS-Glo™ H_2O_2 Assay and CellTox™ Green Cytotoxicity Assay (continued)

4. Apply 40µl of the test compound and CellTox™ Green Dye solution (Step 3) to cells.
5. Measure fluorescence at any point between 0 and 72 hours using an excitation wavelength of 485–500nm and emission of 520–530nm. Adjust the photomultiplier tube (PMT) to optimize the dynamic range. Return the plate to the incubator between reads.
6. After the final CellTox™ Green Assay reading, perform the ROS-Glo™ H_2O_2 Assay according to the homogeneous assay (lytic) protocol (Section 3.D).

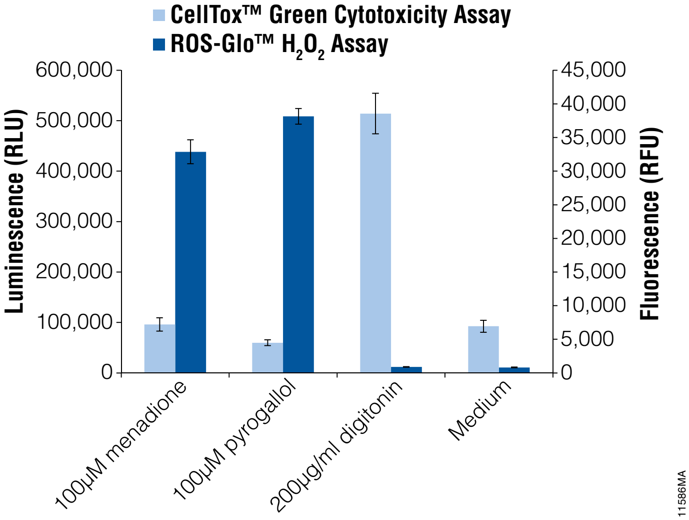

Figure 6. ROS-Glo™ H_2O_2 Assay and CellTox™ Green Cytotoxicity Assay multiplex. HepG2 cells were plated at 2,000 cells/well in a 384-well assay plate and incubated overnight. The cells were then treated with either 100µM menadione, 100µM pyrogallol or 200µg/ml digitonin, and incubated at 37°C in 5% CO_2 for 2 hours. At the dosing time, 1X CellTox™-Green Dye and 25µM H_2O_2 substrate were added to the cell culture. After incubation, the CellTox™ Green fluorescence signal was determined on a Tecan Infinite® M1000 Pro plate reader (excitation 485nm, emission 520nm, bandwidths 5nm). An equal volume of ROS-Glo™ Detection Solution was added to the wells to detect the signal from the ROS-Glo™ H_2O_2 Assay. After a 20-minute incubation at room temperature, the luminescence signal from the ROS-Glo™ Assay was determined on a Tecan Infinite® M1000 Pro plate reader.

Menadione and pyrogallol are ROS-inducing compounds that increase the ROS signal within the 2-hour time frame compared to the medium-only wells. Within these 2 hours there is no significant cytotoxicity observed with these compounds, so the CellTox™ Green signal is low (longer treatments with these drugs would eventually lead to cell death). Digitonin was included as a positive control for cytotoxicity as it is expected to cause cell death within the 2-hour drug incubation time. Due to cell death, the ROS-Glo™ H_2O_2 Assay signal is low.

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Multiplex ROS-Glo™ H_2O_2 Assay and CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Assay detects the ATP content of samples via luminescence and correlates these values to viable cell number. This assay is available from Promega (Cat.# G7570, G7571, G7572, G7573).

1. Perform the ROS-Glo™ H_2O_2 Assay according to the Non-Lytic Assay protocol described in Section 3.D, Step 2.
2. After medium removal for H_2O_2 detection (Step 1 of the Non-Lytic Assay protocol, Section 3.D, Step 2), add an equal volume of the CellTiter-Glo® Reagent to the remaining medium containing cells in the original assay wells. For example, if the assay is set up in 100µl in a 96-well plate, remove 50µl of medium for H_2O_2 detection and add to the ROS-Glo™ Detection Solution in a separate well (as described in Section 3.D, Step 2). Then add 50µl of CellTiter-Glo® Reagent to the remaining 50µl of the reaction mixture in the original assay well.
3. Incubate both assays for 20 minutes at room temperature.
4. Record relative luminescence units using a plate reader for both CellTiter-Glo® Luminescent Cell Viability Assay and the ROS-Glo™ H_2O_2 Assay.

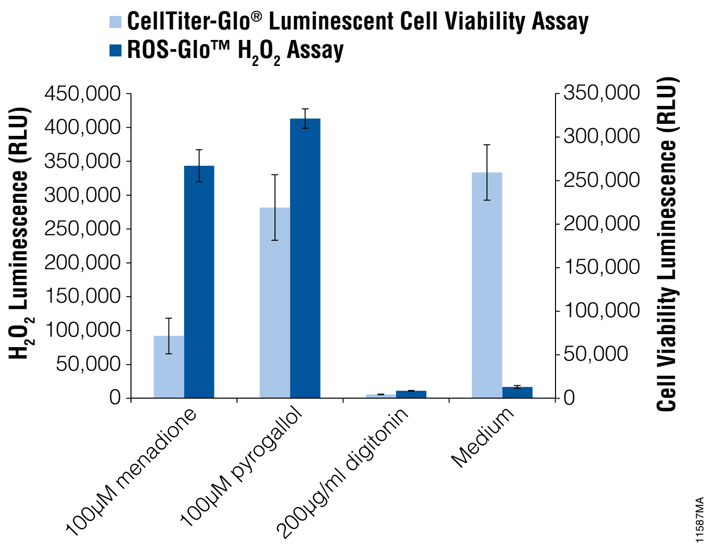

Figure 7. ROS-Glo™ H_2O_2 Assay and CellTiter-Glo® Luminescent Cell Viability Assay multiplex. HepG2 cells were plated at 2,000 cells/well in a 384-well assay plate and incubated overnight. The cells were then treated with either 100µM menadione, 100µM pyrogallol or 200µg/ml digitonin, and incubated at 37°C in 5% CO_2 for 2 hours. H_2O_2 substrate (25µM) was added to the cell culture at the time of dosing. After incubation, an aliquot of medium from the assay wells was removed and added to the ROS-Glo™ Detection Solution in a separate well. The medium that was removed was replaced with PBS. CellTiter-Glo® Reagent was added to the original assay wells in a volume equal to the total volume in the wells. After a 20-minute incubation at room temperature, luminescence signals from the ROS-Glo™ Assay and the CellTiter-Glo® Assay were determined on a Tecan Infinite® M1000 Pro plate reader.

Menadione and pyrogallol are ROS-inducing compounds that increase the ROS signal within the 2-hour time frame compared to the medium-only wells. Because the CellTiter-Glo® Assay detects the level of ATP in viable cells, it can detect the initial drop in ATP levels that occurs when cells are exposed to menadione. With pyrogallol, there is no significant cytotoxicity or drop in ATP levels observed during the 2-hour treatment window; therefore, the CellTiter-Glo® Assay signal remains high (longer treatment with pyrogallol would eventually lead to cell death). Digitonin was included as a positive control for cytotoxicity as it is expected to cause cell death within the 2-hour drug incubation time. Due to cell death, both the ROS-Glo™ H_2O_2 Assay signal and the CellTiter-Glo® Assay signal are low.

5.B. 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
Low signal strength with high concentration of cells	The ROS-Glo™ H_2O_2 Assay is developed for optimal use with 10,000 to 20,000 cells per well in a 96-well plate format. In some cases, the use of high numbers of cells per well results in secondary modifications to the H_2O_2 Substrate that prevent it from generating signal.
	Use fewer cells per well.
	Use a shorter incubation time for H_2O_2 Substrate with cells. For example, incubate H_2O_2 Substrate with cells for 30 minutes instead of 1 hour.
	Perform the experiment with a physiological buffer, such as Hank's Balanced Salt Solution, to minimize unwanted transformations to the H_2O_2 Substrate.
Different background signals in the no-test-compound control samples when using different cell culture media	Some cell culture media contain components that undergo REDOX cycling that produces H_2O_2 in the absence of cells. Other media contain compounds, such as pyruvate, that react directly with hydrogen peroxide and reduce signal. The combination of these effects can change the background signal from the ROS-Glo™ H_2O_2 Assay twofold between media.
	Perform experiments in one medium if possible.
	Run H_2O_2 standards in different medium to allow the actual H_2O_2 level in each medium to be determined.

5.B. Troubleshooting (continued)

Symptoms	Causes and Comments
Lower signals than expected when H_2O_2 is applied to cells	Mammalian cells contain active enzyme systems, such as catalase and glutathione peroxidase, that can rapidly eliminate H_2O_2.
	There is little that can be done to prevent the rapid elimination of H_2O_2 by the cells without altering cellular metabolism. Reducing the number of cells will slow H_2O_2 elimination but at the possible expense of signal generation by other cellular systems.
Increasing the number of cells per well does not increase the ROS-Glo™ H_2O_2 Assay signal	The H_2O_2 level in cell culture is a balance between reactions that generate and eliminate H_2O_2. While increasing cell number may increase H_2O_2 formation, it can also increase H_2O_2 elimination, thus resulting in no net increase in the H_2O_2 concentration.
Low levels of H_2O_2 are observed when a substrate for an H_2O_2-generating enzyme is incubated with a crude extract from cells that contain a significant amount of the enzyme	Catalase and other H_2O_2-eliminating enzyme systems are present in the cell lysate and can eliminate the H_2O_2 made by the enzyme of interest.
	Use a purified enzyme preparation that lacks H_2O_2-eliminating activity.
Increasing background is seen in older assay plates that have a non-binding surface chemistry.	Assay plates, such as the Corning Nonbinding surface (NBS™) plates, use a polyethylene oxide-like surface chemistry that can change over time and result in an elevated background signal from the ROS-Glo™ H_2O_2 Assay.
	Use assay plates that do not have the nonbinding surface, or use fresh nonbinding surface plates for each experiment.
Unexpected inhibition of ROS-Glo™ Assay by test compounds	A luciferase enzyme is used to generate luminescence in the ROS-Glo™ H_2O_2 Assay, so luciferase inhibitors may reduce signals without necessarily affecting H_2O_2 levels. In practice this is rarely observed because luciferase inhibitors are uncommon and the potential for significant luciferase inhibition has been minimized by maintaining high luciferase and ATP concentrations and using a luciferase reaction chemistry that reduces the effects of potential inhibitors.
	To test for luciferase inhibition, apply 400nM Beetle Luciferin, Potassium Salt (Cat.# E1601), to samples of reconstituted Luciferin Detection Reagent. Compare these samples plus and minus the suspected luciferase inhibitor by incubating for 10 minutes at room temperature and then measuring luminescence. If the test compound decreases luminescence, it is a luciferase inhibitor. Luciferase inhibition can be ruled out if no significant difference in signal is observed plus and minus the compound.

6. References

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4. Scott, G.K., *et al*. (2005) Vitamin K3 (menadione)-induced oncosis associated with keratin 8 phosphorylation and histone H3 arylation. *Mol. Pharmacol*. **68**, 606–15.
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7. Babich, H. *et al*. (2009) Choice of DMEM, formulated with or without pyruvate, plays an important role in assessing the in vitro cytotoxicity of oxidants and prooxidant nutraceuticals. *In Vitro Cell Dev. Biol. Anim.* **45**, 226–33.

7. Related Products

Oxidative Stress Assays

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
GSH/GSSG-Glo™ Assay	10ml	V6611
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
Mitochondrial ToxGlo™ Assay	10ml	G8000

Additional sizes available.

Cell Viability, Cytotoxicity and Apoptosis Assays

Product	Size	Cat.#
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® 3D Cell Viability Assay	10ml	G9681
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
RealTime-Glo™ MT Cell Viability Assay	100 assays	G9711
CellTox™ Green Cytotoxicity Assay	10ml	G8741
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
Caspase-Glo® 3/7 Assay System	2.5ml	G8090
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011

Additional sizes available.

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7. Related Products (continued)

Inflammation Assays

Product	Size	Cat.#
Caspase-Glo® 1 Inflammasome Assay	10ml	G9951
Lumit® IFN-γ (Human) Immunoassay	100 assays	W6040
Lumit® IL-1β (Human) Immunoassay	100 assays	W6010
Lumit® IL-2 (Human) Immunoassay	100 assays	W6020
Lumit® IL-4 (Human) Immunoassay	100 assays	W6060
Lumit® IL-6 (Human) Immunoassay	100 assays	W6030
Lumit® IL-10 (Human) Immunoassay	100 assays	W6070
Lumit® TNF-a (Human) Immunoassay	100 assays	W6050

Additional sizes available.

Energy Metabolism Assays

Product	Size	Cat.#
BCAA-Glo™Assay	5ml	JE9300
BHB-Glo™ (Ketone Body) Assay	5ml	JE9500
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190
Dehydrogenase-Glo™ Detection System	5ml	J9010
Glucose-Glo™ Assay	5ml	J6021
Glucose Uptake-Glo™ Assay	5ml	J1341
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Glycogen-Glo™ Assay	5ml	J5051
Lactate-Glo™ Assay	5ml	J5021
Malate-Glo™ Assay	5ml	JE9100
Metabolite-Glo™ Detection System	5ml	J9030
Pyruvate-Glo™ Assay	5ml	J4051
Triglyceride-Glo™ Assay	5ml	J3160

Additional sizes available.

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500

8. Summary of Changes

The following changes were made to the 12/23 revision of this document:

1. Updated Section 7 and patent statements.
2. Changed font and cover image.
3. Made general text edits.

[a]Patents Pending.