TECHNICAL MANUAL

GSH/GSSG-Glo™ Assay

Instructions for Use of Products
V6611 and V6612

Revised 8/15
TM344

GSH/GSSG-Glo™ Assay

All technical literature is available at: www.promega.com/protocols/
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E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Glutathione, a three-amino-acid peptide (gamma glutamyl-cysteinylglycine), is an abundant antioxidant found in eukaryotic cells (1–3). Most of the glutathione exists in reduced form (GSH) in which the sulfhydryl group of the cysteine is not linked in a disulfide linkage to a second glutathione. A small percentage of the glutathione is oxidized and present as a dimer of two of the peptide elements connected by a disulfide bond between the cysteines present in both molecules. Oxidized glutathione (GSSG) is an indicator of cell health and oxidative stress. Certain chemicals react with GSH to form adducts or to increase the GSSG levels, decreasing the ratio of reduced to oxidized glutathione (GSH/GSSG). Measurements of both GSH and GSSG are useful in experimental systems because changes in the GSH/GSSG ratio are associated with human disease, aging and cell signaling events (4–6).

The GSH/GSSG-Glo™ Assay[a,b] is a luminescence-based system to detect and quantify total glutathione (GSH +GSSG), GSSG and GSH/GSSG ratios in cultured cells. The assay provides a simple, rapid multiwell-plate format where stable luminescent signals are correlated with either the GSH or GSSG concentration of a sample. Both GSH and GSSG determinations are based on the reaction scheme shown in Figure 1 where GSH-dependent conversion of a GSH probe, Luciferin-NT, to luciferin by a glutathione S-transferase enzyme is coupled to a firefly luciferase reaction. Light from luciferase depends on the amount of luciferin formed, which in turn depends on the amount of GSH present. Thus, the luminescent signal is proportional to the amount of GSH.

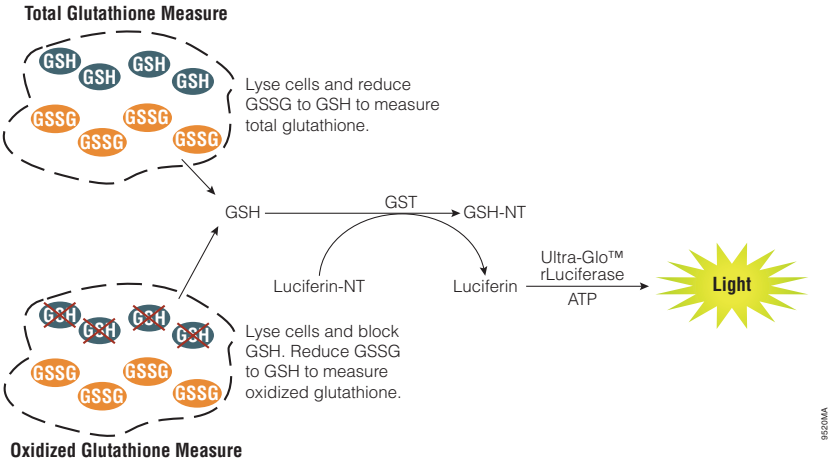

Figure 1. GSH-dependent conversion of a GSH probe, Luciferin-NT, to luciferin by a glutathione S-transferase enzyme is coupled to a firefly luciferase reaction.

In one configuration the reaction scheme is used to measure total glutathione. In this case a reducing agent converts all the glutathione, GSH and GSSG in a cell lysate to the reduced form, GSH. In a second configuration the reaction scheme is used to measure only the oxidized form, GSSG. In this case, a reagent is added that blocks all the GSH while leaving the GSSG intact. This blocking step is followed by a reducing step that converts GSSG to GSH for quantification in the luminescent reaction scheme. The following is a typical assay scenario:

1. Cells in a multiwell plate are treated with test compounds or vehicle: one set of cells for total glutathione measurement and one set for GSSG measurement.
2. Treatments are removed and replaced with a lysis reagent: either Total Glutathione Lysis Reagent for the total glutathione measurement cell set, or Oxidized Glutathione Lysis Reagent for the GSSG measurement cell set.
3. Luciferin Generation Reagent is added to all wells, and assays are mixed and incubated for 30 minutes.
4. Luciferin Detection Reagent is added to all wells, assays are mixed and, after a 15-minute incubation, luminescence is measured.
5. GSH/GSSG ratios are calculated directly from luminescence measurements (in relative light units, RLU) or after interpolation of glutathione concentrations from standard curves.

The assay generates values for total glutathione and GSSG. Since the value for total glutathione represents the signals from both the reduced and oxidized glutathione, subtracting the GSSG reaction signal from the total glutathione signal yields the value of reduced glutathione in the sample.

Advantages of the GSH/GSSG-Glo™ Assay System

Physiologically Relevant GSH/GSSG Ratios: Actual levels of total glutathione and GSSG are measured directly in cell culture wells, minimizing the loss of GSH and GSSG compared to conventional assays that require up-front sample preparation and indirect GSSG calculation.

More Robust Performance: Bioluminescent technology and a simple protocol minimize sample handling, reducing variability.

Simplified Protocol: Assay reagents are added directly to cells cultured in multiwell plates. The homogenous add-mix-read format eliminates time-consuming sample deproteination and centrifugation steps required of conventional assays.

Greater Sensitivity: Fewer cells are required in these assays than in conventional assays due to the enhanced sensitivity.

Faster Results: The homogeneous add-mix-read protocol minimizes hands-on time, and the bioluminescence technology minimizes incubation time.

Adaptable to Automation: The glow-type signal is stable, with a half-life greater than two hours, and the protocol is adaptable to automation.

No Fluorescence Interference: Using luminescence readout eliminates the fluorescent interference between reagents and test compounds sometimes seen in fluorescence assays. Such overlap can confound analysis and present misleading or irrelevant data.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
GSH/GSSG-Glo™ Assay	**10ml**	**V6611**

Each system contains sufficient reagents to perform a total of 100 reactions: 100 total glutathione reactions, 100 GSSG reactions or 50 of each reaction for GSH/GSSG ratio determination in a 96-well format (not including control or standard curve reaction wells). Includes:

- 50µl NEM, 25mM
- 1.0ml Passive Lysis Buffer, 5X
- 250µl DTT, 100mM
- lyophilized Luciferin Detection Reagent
- 10ml Reconstitution Buffer with Esterase
- 10ml Glutathione Reaction Buffer
- 100µl Luciferin-NT
- 100µl Glutathione, 5mM
- 500µl Glutathione S-Transferase

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
GSH/GSSG-Glo™ Assay	**50ml**	**V6612**

Each system contains sufficient reagents to perform a total of 500 reactions: either 500 total glutathione reactions, 500 GSSG reactions or 250 of each reaction for GSH/GSSG ratio determination in a 96-well format (not including control or standard curve reaction wells). Includes:

- 250µl NEM, 25mM
- 5ml Passive Lysis Buffer, 5X
- 1.25ml DTT, 100mM
- lyophilized Luciferin Detection Reagent
- 50ml Reconstitution Buffer with Esterase
- 50ml Glutathione Reaction Buffer
- 500µl Luciferin-NT
- 2 × 100µl Glutathione, 5mM
- 3 × 500µl Glutathione S-Transferase

Storage Conditions: Store all components at –20°C protected from light. The reconstituted Luciferin Detection Reagent can be stored at room temperature (approximately 22°C) for 1 week or –20°C for 2 months with no change in activity. **Prepare Total and Oxidized Glutathione Lysis Reagents and Luciferin Generation Reagent immediately before use. Do not store these reagents for future use.**

Luciferin-NT substrate, Glutathione Reaction Buffer and Glutathione S-Transferase are stable at room temperature for several hours as separate solutions. Do not thaw the solutions above 25°C, and mix well after thawing. The most convenient and effective method for thawing is to place the reagent in a room-temperature water bath.

3. Performing the GSH/GSSG-Glo™ Assay

3.A. General Considerations

Measurement of total glutathione (GSH + GSSG) or oxidized glutathione (GSSG) is accomplished after cell lysis.

Medium removal is necessary before lysis when assaying adherent cells because components of cell culture media such as serum, cysteine and glutathione interfere with accurate measurement of intracellular GSH and GSSG.

Cell lysis of adherent or suspended mammalian cells is accomplished with Passive Lysis Buffer that contains Luciferin-NT, the luminogenic GSH probe required for the assay chemistry. Two lysis reagents are:

Total Glutathione Lysis Reagent. This lysis reagent releases the reduced and oxidized glutathione and converts all glutathione to the reduced form of GSH.

Oxidized Glutathione Lysis Reagent. This lysis reagent releases reduced and oxidized glutathione and contains N-Ethylmaleimide (NEM), which reacts with GSH to produce a form that cannot contribute to luminescent signal. Cell lysis and GSH blockage by NEM is extremely rapid, taking place within one minute after adding lysis reagent.

Be sure to use the correct formulation of the **Glutathione Lysis Reagent** for the glutathione measurement desired. Using the incorrect formulation will result in incorrect measurement of the desired form of glutathione.

Luciferin Generation Reagent. This reagent generates one mole of luciferin from the GSH probe, Luciferin-NT, by the action of GST for each mole of reduced glutathione present in the reaction.

Note: In a GSSG measurement two moles of GSH and, in turn, two moles of luciferin are formed per mole of GSSG. Therefore, light corresponding to a mole of GSSG is two times brighter than light from a mole of GSH.

Luciferin Detection Reagent. This reagent is a luciferase formulation that creates light in direct proportion to the amount of luciferin produced from the GSH probe, Luciferin-NT. This reagent simultaneously stops the Luciferin Generation Reaction and initiates a luminescent signal that is directly proportional to the amount of GSH derived from total glutathione (GSH + GSSG) or GSSG alone. The proprietary stabilized luciferase (Ultra-Glo™ Luciferase) produces a stable "glow-type" luminescent signal with a half-life greater than two hours. This eliminates the need for strictly timed luminescence detection.

Tips for Success

- Include a vehicle (or untreated) control and a background control (no cells) on each plate. The vehicle control enables observation of solvent effects on GSH/GSSG. The no-cells control gives a measure of background signal from the assay chemistry, which can be subtracted from vehicle and test signals to give net values.
- To achieve linear assay performance at low light levels, the background luminescence (no-cells control) must be subtracted from all readings. Some instruments also require verification of linear response at high light levels.
- A period of 15 minutes is required for luminescence to stabilize after adding Luciferin Detection Reagent and before reading with a luminometer. The reagent is not designed for use with the automated reagent injectors that are integrated into some luminometers.
- Use opaque, white multiwell plates that are compatible with your luminometer. Light signals are diminished in black plates, and well-to-well cross talk is observed in clear plates.
- Use an integration time of 0.25–1 second per well as a luminometer guideline. Relative light units are arbitrary units that vary depending on the instrument manufacturer and model. Absolute readings from one luminometer may not match those from another model. Consult the luminometer instructions for proper use of the instrument.
- Firefly luciferase activity is optimal at room temperature (20–25°C); equilibrate reagents to room temperature before measuring luminescence.

3.B. Reagent Preparation

Materials to Be Supplied by the User

(Solution compositions are provided in Section 5.A.)

- distilled or deionized water
- white, opaque, polystyrene, flat-bottom 96-well plates
- luminometer capable of reading multiwell plates
- multichannel pipette or automated repeating pipettor

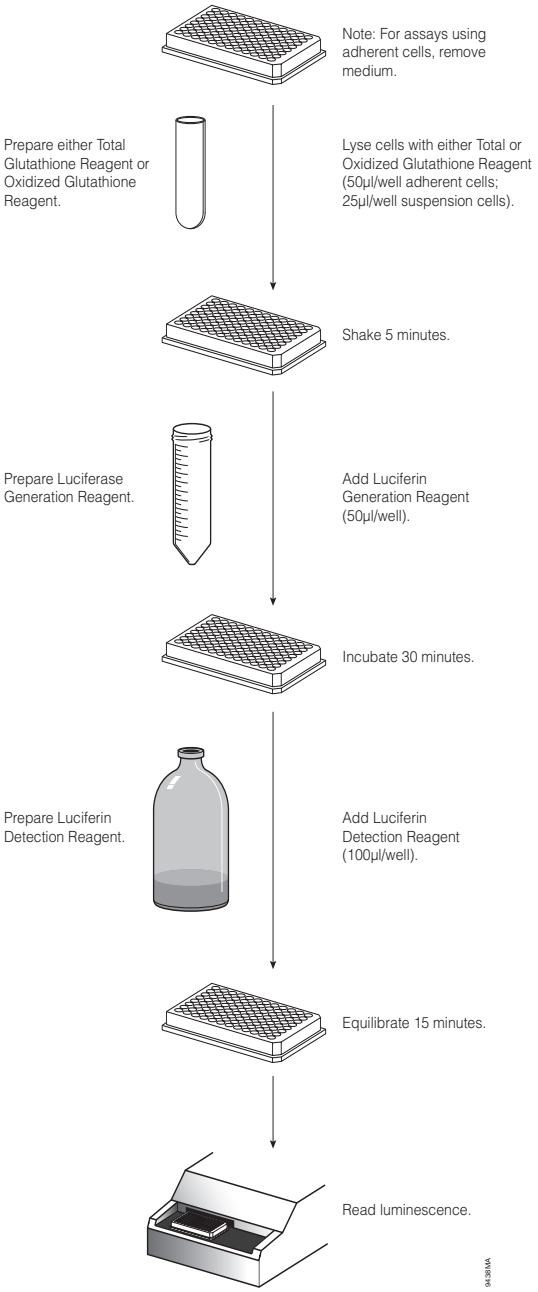

Figure 2. Workflow for the GSH/GSSG-Glo™ Assay.

3.B. Reagent Preparation (continued)

Assay Format for Adherent Cells
50µl Glutathione Lysis Reagent
50µl Luciferin Generation Reagent
100µl Luciferin Detection Reagent

Reagents for Assays with Adherent Cells

Total Glutathione Lysis Reagent

Component	Volume per Reaction (96-well plate)
Luciferin-NT	1.0µl
Passive Lysis Buffer, 5X	10.0µl
Water	39.0µl
Final volume per reaction	**50.0µl**

Note: Use the Total Glutathione Lysis Reagent within 30 minutes of preparing it.

Oxidized Glutathione Lysis Reagent

Component	Volume per Reaction (96-well plate)
Luciferin-NT	1.0µl
NEM, 25mM	0.5µl
Passive Lysis Buffer, 5X	10.0µl
Water	38.5µl
Final volume per reaction	**50.0µl**

Note: Use the Oxidized Glutathione Lysis Reagent within 30 minutes of preparing it.

Luciferin Generation Reagent

Component	Volume per Reaction (96-well plate)
100mM DTT	1.25µl
Glutathione-S-Transferase	3.0µl
Glutathione Reaction Buffer	45.75µl
Final volume per reaction	**50.0µl**

Note: Adjust the total volume of Lysis and Luciferin Generation Reagents for the total number of reactions that you will perform, including vehicle and no-cell controls and standard curve assays.

Luciferin Detection Reagent: Transfer the contents of one bottle of Reconstitution Buffer with Esterase to the amber bottle of lyophilized Luciferin Detection Reagent. Mix by inversion until the substrate is thoroughly dissolved. Do not vortex. Volume per reaction (in a 96-well plate): 100µl.

3.B. Reagent Preparation (continued)

Reagents for Assays with Suspension Cells

When suspension cell cultures are assayed, the buffer containing the test compound used to treat the cells is not removed. The lysis reagents are prepared in a more concentrated form and are added directly to the cell solution.

Assay Format for Suspension Cells
25µl suspension cells plus test compound or vehicle
25µl Glutathione Lysis Reagent
50µl Luciferin Generation Reagent
100µl Luciferin Detection Reagent

Total Glutathione Lysis Reagent (concentrated)

Component	Volume per Reaction (96-well plate)
Luciferin-NT	1.0µl
Passive Lysis Buffer, 5X	10.0µl
Water	14.0µl
Final volume per reaction	**25.0µl**

Note: Use the Total Glutathione Lysis Reagent (concentrated) within 30 minutes of preparing it.

Oxidized Glutathione Lysis Reagent (concentrated)

Component	Volume per Reaction (96-well plate)
Luciferin-NT	1.0µl
NEM, 25mM	0.5µl
Passive Lysis Buffer, 5X	10.0µl
Water	13.5µl
Final volume per reaction	**25.0µl**

Note: Use the Oxidized Glutathione Lysis Reagent within 30 minutes of preparing it.

Luciferin Generation Reagent

Component	Volume per Reaction (96-well plate)
100mM DTT	1.25µl
Glutathione-S-Transferase	3.0µl
Glutathione Reaction Buffer	45.75µl
Final volume per reaction	**50.0µl**

Note: Adjust the total volume of Lysis and Luciferin Generation Reagents for the total number of reactions that you will perform, including vehicle and no-cell controls and standard curve assays.

Luciferin Detection Reagent: Transfer the contents of one bottle of Reconstitution Buffer with Esterase to the amber bottle of lyophilized Luciferin Detection Reagent. Mix by inversion until the substrate is thoroughly dissolved. Do not vortex. Volume per reaction (in a 96-well plate): 100µl.

Glutathione Standard Curve, 0–16µM

Inclusion of a GSH standard curve allows conversion of luminescence (in RLU) to GSH and GSSG concentrations. Standard curves are most important when optimizing cell densities; it is not necessary to perform a standard curve with every experiment.

Dilute 5mM Glutathione to 320µM in water. For example, dilute 32µl of 5mM GSH with 468µl water. Perform 1:2 serial dilutions by transferring 250µl of diluted GSH to 250µl water. **This assay will not be linear over the entire range of concentrations. Use the linear portion of the curve to calculate concentrations or use curve-fitting software.**

Transfer 5µl per well of each GSH dilution to empty wells of the 96-well assay plate. For the standard curve, 5µl of 320µM GSH corresponds to a final concentration of 16µM. See Figure 3 legend and the suggested plate layout in the Data Analysis section.

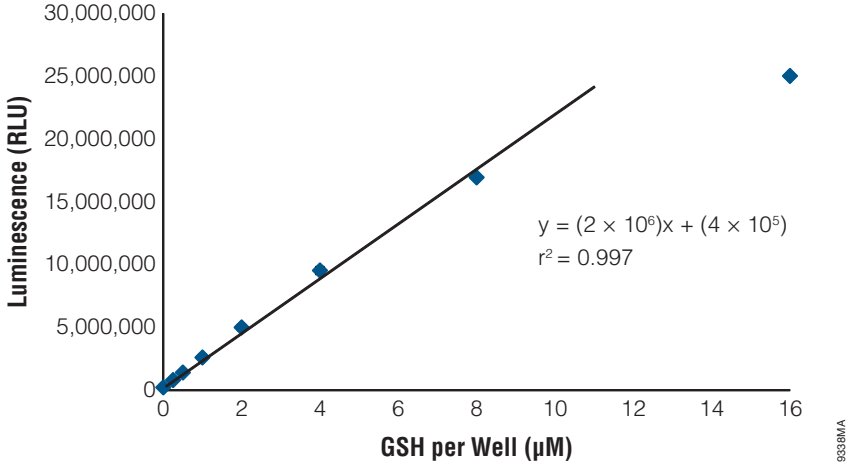

Figure 3. Glutathione standard curve. A standard curve was generated by serial twofold dilutions of 320µM glutathione. Five microliters of each dilution was added to triplicate wells containing 50µl of Total Glutathione Lysis Reagent. After mixing, an equal volume (50µl) of Luciferin Generation Reagent was added to each well, and the plate was incubated for 30 minutes at room temperature. An equal volume (100µl) of Luciferin Detection Reagent was added to each well, and after 15 minutes at room temperature, luminescence was measured using a luminometer. The linear portion of this curve (0–8µM GSH) was used for calculations.

Notes:

1. Only the Total Glutathione Lysis Reagent may be used to generate the glutathione standard curve as the Oxidized Glutathione Lysis Reagent contains NEM and would modify or inactivate GSH.
2. Prepare GSH Standard Curve dilutions no more than 30 minutes before performing the assay.

3.C. Recommended Controls

No-cell control. This control reflects the assay background. Set up wells with medium and experimental treatment but no cells.

Vehicle control. This is a control for solvent effects on the assay chemistry or cells. It also provides the "basal" level of activity for the cells (i.e., activity in the absence of experimental treatment). Set up wells with medium, cells and vehicle used to deliver experimental treatment but no experimental treatment.

3.D. Assay Procedure for Measuring GSSG and Total Glutathione in Adherent Mammalian Cells

Plating Cells

Plate cells and allow to grow overnight at 37°C in a 5% CO_2 culture incubator.

The assay is optimized for low cell densities such as HeLa cells plated at 5,000–10,000 cells/well and hepatocytes at 10,000–20,000 cells/well. Plate cells at the desired density in 96-well luminometer-compatible tissue culture plates. Consider assaying cells at different densities to determine the optimum for brightness, while still obtaining values within the linear region of the glutathione standard curve (Section 3.B).

Consider plating cultured cell lines on flat-bottom, tissue culture-treated white-with-clear-bottom 96-well plates such as Corning Costar® 3903. Hepatocytes can be cultured on white-walled, collagen-coated plates with clear bottoms (e.g., BD BioCoat™ 4650 96-well Collagen 1 Cellware). If clear-walled tissue culture plates are used, an additional step must be performed after adding Luciferase Detection Reagent to transfer the reactions into luminometer-compatible plates.

Treating Cells

Components of cell culture media, including glutathione, serum and phenol red, may interfere with the assay chemistry. Remove growth medium and replace with test compounds in a physiological buffer such as Hank's Balanced Salts, Krebs-Ringer Bicarbonate Buffer or Kreb's Henseleit Buffer. No wash step is needed before adding test compounds.

Note: For vehicle controls, replace the growth medium with buffer containing vehicle at the same time cells are treated with test compounds.

Assay Procedure for Adherent Cells

1. Remove and discard buffer containing test compound and/or vehicle from the cells. Add 50µl per well of Total Glutathione Lysis Reagent or Oxidized Glutathione Lysis Reagent, as appropriate for desired endpoint. Prepare the Lysis Reagent no longer than 30 minutes before use.

   Add 50µl of Total Glutathione Lysis Reagent to the wells containing no cells (no-cell controls) and to the wells containing the glutathione standard curve.

   Shake the plate at room temperature for 5 minutes on a plate shaker.

2. Add 50µl per well of Luciferin Generation Reagent to all wells.

   Note: Use the Luciferin Generation Reagent within 30 minutes of preparation.

3. Shake plate briefly, and incubate at room temperature for 30 minutes.

4. Add 100µl per well of Luciferin Detection Reagent. Shake plate briefly, wait 15 minutes and measure luminescence.

3.E. Assay Procedure for Measuring GSSG and Total Glutathione in Cells Grown in Suspension

Grow cells, and centrifuge, count and dilute cells to desired density in Krebs-Ringer, Krebs-Henseleit or Hank's Balanced Salts.

Note: For most cells, use a cell density of $1–5 \times 10^5$ cells/ml. You may need to optimize cell density for particular cell types. Choose the cell density that allows detection of glutathione within the linear region of the standard curve (Section 3.B).

Assay Procedure for Cells in Suspension

1. To wells of a 96-well luminometer-compatible plate, add 20µl per well of cells in an appropriate buffer or medium such as Krebs-Ringer, Krebs-Henseleit, or Hank's Balanced Salts. Prepare enough wells for cells treated with test compound, cells with vehicle only (vehicle control) and no-cell controls.

2. Add 5µl per well of the test compound and vehicle at 5X concentration.

   If you are preparing a standard curve, during the incubation, add 5µl of each glutathione standard dilution to empty wells of a 96-well plate as described in Section 3.B for the standard curve.

3. Add 25µl per well Total Glutathione Lysis Reagent (concentrated) or Oxidized Glutathione Lysis Reagent (concentrated), depending on desired measurement, to the wells containing cells.

   Note: Add 20µl of buffer or medium and 25µl of Total Glutathione Lysis Reagent to the wells containing glutathione standards. Use Lysis Reagent within 30 minutes of preparation.

4. Shake the plate for 5 minutes on a plate shaker.

5. Add 50µl per well of Luciferin Generation Reagent to all wells. Prepare the Luciferin Generation Reagent immediately before use.

6. Shake briefly, and incubate at room temperature for 30 minutes.

7. Add 100µl per well of Luciferin Detection Reagent. Shake briefly, wait 15 minutes and measure luminescence.

4. Data Analysis

To calculate the GSH/GSSG ratio, two things are required: total glutathione measurement and GSSG measurement. (**Note:** A twofold adjustment is required for GSSG because each mole of oxidized GSSG upon reduction in this assay produces two moles of GSH.)

Calculating the Ratio from GSH Standard Curve

1. Plot net RLU v. µM GSH. Make a second plot with the x-axis values divided by two to reflect the micromolar GSSG concentration.

   Note: Two moles of GSH are generated per 1 mole of GSSG. Dividing the GSH concentrations by two gives the concentration of GSSG.

2. Using the slope (m) generated by the linear portion of each standard curve and the formula y = mx + b, convert the average net RLU values of treated and untreated cells to micromoles of GSSG and GSH.

3. Subtract the GSSG contribution to the total glutathione concentration by multiplying the micromole GSSG values by 2 and subtracting this value from the total glutathione amount to generate the GSH concentration in the samples.

4. $$\text{Ratio GSH/GSSG control} = \frac{\mu\text{M total glutathione vehicle} - (\mu\text{M GSSG vehicle} \times 2)}{\mu\text{M GSSG vehicle}}$$

5. $$\text{Ratio GSH/GSSG treated} = \frac{\mu\text{M total glutathione treated} - (\mu\text{M GSSG treated} \times 2)}{\mu\text{M GSSG treated}}$$

Sample Calculation

Total Glutathione Data

Sample	net RLU	µM GSH
Std 1	35,051,219	16
Std 2	22,964,239	8
Std 3	13,054,213	4
Std 4	6,611,359	2
Std 5	3,365,347	1
Std 6	1,690,381	0.5
Std 7	833,584	0.25
zero	0	0
vehicle	14,989,339	5.177
treated	12,364,719	4.271

GSSG Data

Sample	net RLU	µM GSSG
Std 1	35,051,219	8
Std 2	22,964,239	4
Std 3	13,054,213	2
Std 4	6,611,359	1
Std 5	3,365,347	0.5
Std 6	1,690,381	0.25
Std 7	833,584	0.125
zero	0	0
vehicle	455,294	0.079
treated	5,894,363	1.018

Note: Grayed boxes in the sample calculation data table indicate the linear portion of the standard curve for this particular experiment.

$$\text{Ratio GSH/GSSG (vehicle)} = \frac{5.177 - (0.079 \times 2)}{0.079} = 63.5$$

$$\text{Ratio GSH/GSSG (treated)} = \frac{4.271 - (1.018 \times 2)}{1.018} = 2.2$$

Calculating the Ratio from Net RLU (without a standard curve)

1. **Background:** Use the luminescence (in RLU) from no-cells control or luminescence from the 0µM GSH from the standard curve.
2. **Cells with Test Compound and Vehicle:** Subtract the average background luminscence (in RLU) from all wells. The result represents the net luminescence (in RLU) for the samples.
3. Calculate the GSH/GSSG ratio for vehicle-control cells using this formula:

$$\frac{\text{(Net vehicle total glutathione RLU} - \text{Net vehicle GSSG RLU)}}{\text{[Net vehicle GSSG RLU/2]}}$$

4. Calculate the GSH/GSSG ratio for treated cells:

$$\frac{\text{(Net treated total glutathione RLU} - \text{Net treated GSSG RLU)}}{\text{[Net treated GSSG RLU/2]}}$$

The signal from the GSSG reaction in the denominator is divided by 2 to reflect the number of moles of GSSG in the sample, not the number of moles of GSH generated from the oxidized glutathione reaction.

The signal from a mole of GSSG will appear as the signal from two moles of GSH; therefore, the measured value for the oxidized glutathione is divided by two.

Sample Calculation

Total Glutathione Data

Sample	**Net RLU**
Vehicle Control	14,989,339
Treated	12,364,719

GSSG Data

Sample	**Net RLU**
Vehicle Control	455,294
Treated	5,894,363

$$\text{Ratio GSH/GSSG (vehicle)} = \frac{14{,}989{,}339 - 455{,}294}{455{,}294/2} = 63.8$$

$$\text{Ratio GSH/GSSG (treated)} = \frac{12{,}364{,}719 - 5{,}894{,}363}{5{,}894{,}363/2} = 2.2$$

5. Appendix

5.A. Composition of Buffers and Solutions

Glutathione Reaction Buffer

50mM Tricine (pH 7.9)

5.B. Plate Layout

	Cells: Assay for GSSG				Cells: Assay for total glutathione				No cells: GSH Standard Curve			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Test Compound				Test Compound				16µM			
B	Vehicle				Vehicle				8µM			
C									4µM			
D									2µM			
E									1µM			
F									0.5µM			
G									0.25µM			
H									0			

Notes:

1. The 0µM wells of the standard curve can serve as the no-cell controls.
2. Menadione may be used as a positive control (to increase GSSG) for many cells (7).

5.C. References

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6. Ghezzi, P. (2005) Regulation of protein function by glutathionylation. *Free Radic. Res*. **39**, 573–80.
7. Sun, J.S. *et al*. (1997) Menadione-induced cytotoxicity to rat osteoblasts. *Cell Mol. Life Sci*. **53**, 967–76.

5.D. Sample Data

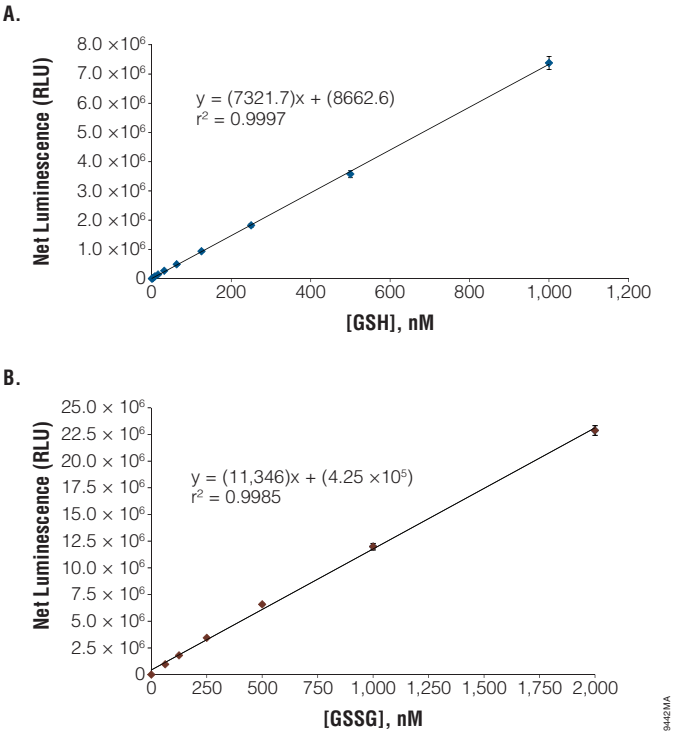

Figure 4. Limit of dectection using reduced and oxidized glutathione. GSH and GSSG standards were assayed using the GSH/GSSG-Glo™ System and a GloMax® Luminometer. The results were averaged and plotted on the graphs above. A linear trend line was generated and correlation coefficients calculated for the fit of the data to the trend line. Limits of detection were 2.5nM GSH and 0.5nM GSSG.

Table 1. GSH/GSSG Ratios from Various Cell Types.

		GSH/GSSG Ratio	
Cell Type	**Cells/Well**	**Vehicle Control Cells**	**Menadione-Treated Cells**
HeLa	5,000	65.8	4.2
Rat Hepatocytes	20,000	21.8	3.1
HepG2	5,000	75.2	3.5
Jurkat	10,000	15.0	3.8

Cells were assayed using the suspension or adherent cell protocol in 96-well plates, and the values obtained were used to calculate the GSH/GSSG ratio. Cells were treated with 40µM menadione or 0.1% DMSO for 60 minutes at 37°C in a tissue culture incubator.

5.E. 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	Possible Causes and Comments
Glutathione standard curve not linear	The glutathione standard curve should be linear to 8µM. Adjust gain settings on the luminometer to accommodate brighter signals. Dilute test samples to bring signals within linear range of instrument.
	Linear regression showed r^2 <0.97, resulting in higher than targeted GSH concentrations. Check that GSH dilutions were correct. The final GSH standard concentrations, after adding the Luciferin Generation Reagent, should be 16, 8, 4, 2, 1, 0.5, and 0.25µM.
Low signal from glutathione standard curve	Be sure that Total Glutathione Lysis Reagent was used (not Oxidized Glutathione Lysis Reagent).
High background luminescence	Luciferin contamination in one or more of the reaction components: • Avoid workspaces and pipettes that are used with luciferin-containing solutions, including luminescence-based cell viability, apoptosis or gene reporter assays. • Decontaminate work surfaces by wiping with a detergent solution or ethanol and rinsing with clean water. Rinse pipettes and other labware with distilled water at least three times. For automated dispensing systems, replace any components that have been used to dispense luciferin-containing solutions.
	Substrate was stored improperly. Luciferin-NT should be stored at –20°C, protected from light.
	Glutathione Lysis Reagents were prepared and stored before use. Prepare Glutathione Lysis Reagents no longer than 30 minutes before use. Do not store reagent for future (or further) use.
Low luminescent signal	Use only white, opaque luminometer plates. Do not use black plates or clear plates.
	Avoid multiple freeze-thaw cycles of the reconstituted Luciferin Detection Reagent.

Symptoms	Possible Causes and Comments
Unexpected inhibition of luciferase or GST enzyme	The GST enzyme catalyzes the luciferin-generating reaction, and a luciferase enzyme is used to generate luminescence in the luciferin-detection step of the GSH/GSSG-Glo™ Assay. The potential for inhibition of luciferase or GST enzyme is minimized by high enzyme concentrations and reaction chemistries that reduce the effects of potential inhibitors.
	To screen for luciferase inhibition, assemble two reactions, one with equal volumes of reconstituted Luciferin Detection Reagent and 400nM Beetle Luciferin, Potassium Salt (Cat.# E1601) in water, and a second reaction with equal volumes of reconstituted Luciferin Detection Reagent and 400nM beetle luciferin plus the test compound. Incubate reactions for 10 minutes at room temperature, and then measure luminescence. A decrease in luminescence in the presence of test compound is an indication of luciferase inhibition.
	If luciferase inhibition was ruled out, perform the GSH/GSSG-Glo™ Assay with and without test compound and 5µM glutathione. Diminished signal in the reaction with test compound compared to reaction without test compound indicates GST enzyme inhibition.
Half-life of signal was less than two hours	Cell density was too high. Repeat the assay using lower cell densities until a suitable signal stability is established.

5.F. Related Products

Product	Size	Cat.#
GSH-Glo™ Glutathione Assay	10ml	V6911
	50ml	V6912

Luciferin

Product	Size	Cat.#
Beetle Luciferin, Potassium Salt	5mg	E1601
	50mg	E1602
	250mg	E1603
	1g	E1605

Cell Viability, Cytotoxicity and Apoptosis Assays

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
CellTiter-Fluor™ Cell Viability Assay	10ml	G6080
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	200 assays	G3582
CellTiter-Blue® Cell Viability Assay	20ml	G8080
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CytoTox-Fluor™ Cytotoxicity Assay	10ml	G9260
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
MultiTox-Glo Multiplex Cytotoxicity Assay	10ml	G9270
Caspase-Glo® 3/7 Assay	100ml	G8092
Caspase-Glo® 8 Assay	100ml	G8202
Caspase-Glo® 9 Assay	100ml	G8212
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml	G7791
Caspase-Glo® 2 Assay	50ml	G0941
Caspase-Glo® 6 Assay	50ml	G0971

Available in Additional Sizes.

Mechanism-Based Toxicity Assays

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

Available in Additional Sizes.

ADME Assays

P-Glycoprotein Assays

Product	Size	Cat.#
Pgp-Glo™ Assay System	10ml	V3591
Pgp-Glo™ Assay System with P-glycoprotein	10ml	V3601

Monoamine Oxidase Assay

Product	Size	Cat.#
MAO-Glo™ Assay	200 assays	V1401

Available in Additional Sizes.

Cytochrome P450 Assays

Product	Size	Cat.#
P450-Glo™ CYP2B6 Assay	10ml	V8321
P450-Glo™ CYP1A2 Induction/Inhibition Assay	10ml	V8421
P450-Glo™ CYP1A1 Assay*	10ml	V8751
P450-Glo™ CYP1B1 Assay*	10ml	V8761
P450-Glo™ CYP1A2 Assay*	10ml	V8771
P450-Glo™ CYP2C8 Assay*	10ml	V8781
P450-Glo™ CYP2C9 Assay*	10ml	V8791
P450-Glo™ CYP3A4 Assay*	10ml	V8801
P450-Glo™ CYP3A7 Assay*	10ml	V8811
P450-Glo™ CYP2C19 Assay*	10ml	V8881
P450-Glo™ CYP2D6 Assay*	10ml	V8891
P450-Glo™ CYP3A4 (Luciferin-PFBE) Cell-Based/Biochemical Assay*	10ml	V8901
P450-Glo™ CYP3A4 (Luciferin-PPXE) DMSO-Tolerant Assay*	10ml	V8911

*Available in Additional Sizes.

5.F. Related Products (continued)

Cytochrome P450 Assays (continued)

Product	Size	Cat.#
P450-Glo™ CYP3A4 Assay with Luciferin-IPA	10ml	V9001
P450-Glo™ CYP1A2 Screening System	1,000 assays	V9770
P450-Glo™ CYP2C9 Screening System	1,000 assays	V9790
P450-Glo™ CYP3A4 Screening System	1,000 assays	V9800
P450-Glo™ CYP2C19 Screening System	1,000 assays	V9880
P450-Glo™ CYP2D6 Screening System	1,000 assays	V9890

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System	1 each	GM3500
GloMax® 96 Microplate Luminometer	1 each	E6501
GloMax® 20/20 Luminometer	1 each	E5311

6. Summary of Changes

The following changes were made to the 8/15 revision of this document:

1. The document design was updated.
2. Related products were updated.

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294, European Pat. No. 1131441 and other patents pending.

[c]Certain applications of this product may require licenses from others.