DETECTING TOXICOLOGICAL RESPONSES IN CELLS WITH THE BIOLUMINESCENT GSH-GLO™ GLUTATHIONE ASSAY

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Here we present a new bioluminescent assay for determining the concentration of the reduced form of glutathione in cells or tissue lysates. The bioluminescent format reduces test compound interference and uses a protocol easily amenable to high-throughput screening applications.

Introduction

Glutathione (GSH), a tripeptide (L γ-glutamyl-cysteinyl-glycine) is the major nonprotein thiol found abundantly in eukaryotic cells (1–3). GSH plays a critical role in many metabolic pathways. It acts as a cofactor for intracellular enzymes like the glutathione S-transferases (GST) and glutathione peroxidase; it aids in amino acid transport and is essential for detoxification of xenobiotics and reactive oxygen species (ROS). GSH is also involved in maintenance of cellular redox-regulated signal transduction. Reactive oxygen species and unintended drug-drug interactions often cause a drop in GSH levels by reaction

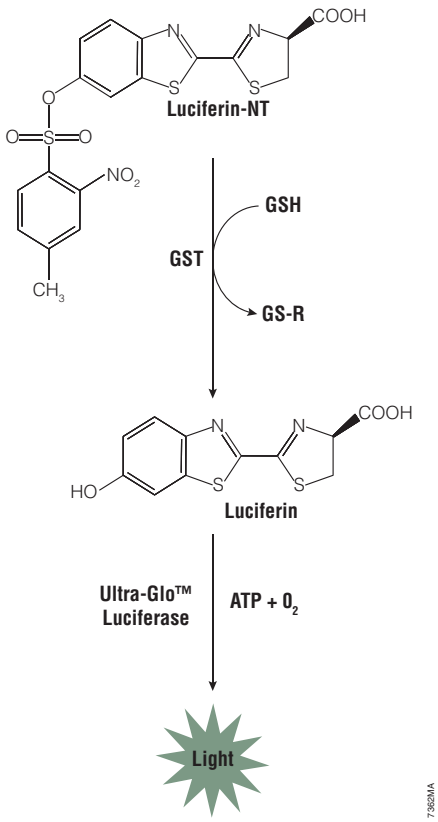

Figure 1. Schematic of the GSH-Glo™ Glutathione Assay. The assay chemistry occurs in two steps. First cells are lysed in the presence of the proluminogenic Luciferin-NT substrate and glutathione S-transferase; GSH in the cells drives the formation of luciferin. Next, the Luciferin Detection Reagent is added, and the Ultra-Glo™ Luciferase acts on the luciferin, releasing light as a by product of the reaction. The amount of light released is proportional to the concentration of reduced GSH in the cells.

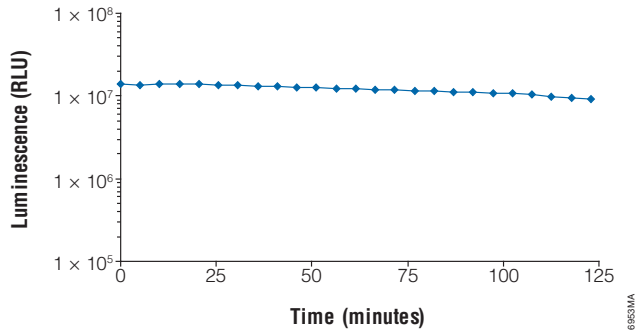

Figure 2. Steady-state luminescence. GSH (5 µM) was added to 100 µl of GSH-Glo™ reaction mix and incubated for 30 minutes. The reaction was terminated by adding 100 µl of Luciferin Detection Reagent and monitored over time using the GloMax® 96 Microplate Luminometer (Cat.# E6501). The half-life of the GSH-Glo™ signal was calculated to be greater than 2 hours under assay conditions.

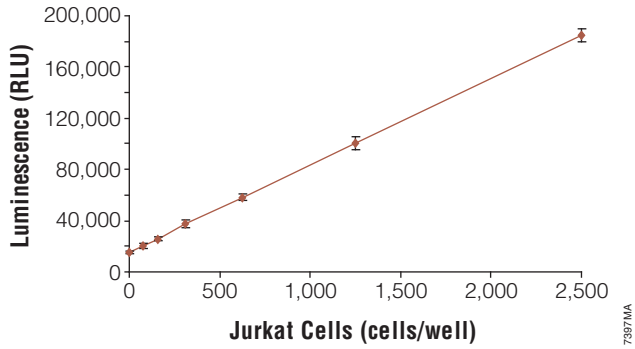

Figure 3. The GSH-Glo™ Assay demonstrates a linear response to a serial titration of cells. Suspension cells (Jurkat cells) were harvested, counted and resuspended in PBS. A serial twofold dilution of cells in PBS was then prepared directly in a 384-well plate. An equal volume of GSH-Glo™ Reagent (2X) was added to the wells and incubated for 30 minutes. The GSH-Glo™ Assay reaction was stopped by adding an equal volume of reconstituted Luciferin Detection Reagent, and luminescence was read after 15 minutes. Based on the GSH standard curve, the limit of detection (LOD) for Jurkat cells was approximately 20 cells. The r^2 value was determined to be 0.999.

Detect GSH with a Bioluminescent Assay

Table 1. Comparison of GSH-Glo™ Assay with a Typical Fluorescent Assay for Measuring Glutathione in Biological Samples.

GSH-Glo™ Assay Protocol	Fluorescent Assay Protocol
✔ Plate cells at desired density in multiwell culture plate.	✔ Obtain 1 × 10^6–10^8 cells.
✔ Treat cells with test compound (optional).	✔ Treat the cells with test compound (optional).
✔ Prepare the GSH-Glo™ Reagent as directed.	✔ Harvest cells and centrifuge.
✔ Prepare a GSH standard curve (optional).	✔ Wash cells with PBS.
✔ Remove growth medium from the wells of the multiwell plate.*	✔ Centrifuge cells.
✔ Add the GSH-Glo™ Reagent.	✔ Resuspend the cells, lyse and remove protein from the sample.
✔ Incubate for 30 minutes.	✔ Centrifuge the lysate.
✔ Add the Luciferin Detection Reagent.	✔ Neutralize lysate.
✔ Incubate 15 minutes.	✔ Measure volume and dilute lysate as appropriate.
✔ Read luminescence.	✔ Transfer lysate to wells of a multiwell plate.
	✔ Prepare GSH standard curve.
	✔ Transfer assay substrate to wells.
	✔ Transfer enzyme-regenerating solution to wells.
*The example provided here is for adherent cells.	✔ Read fluorescence multiple times over 30 minutes.

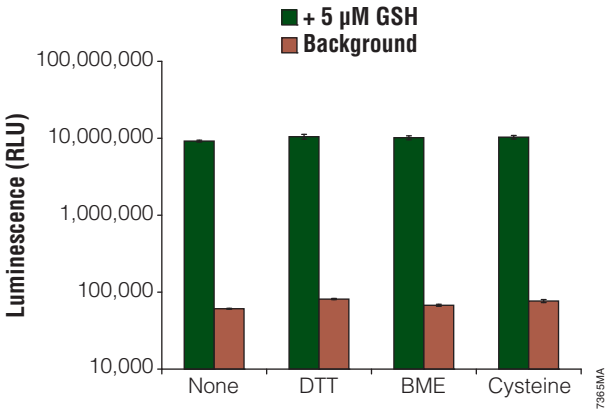

Figure 4. Specificity of Luciferin-NT substrate. GSH (5 µM) was added to reaction mixes containing 1 mM of three common reducing agents: dithiothreitol (DTT), β-mercaptoethanol (BME) or cysteine. The vehicle-only control contained GSH reaction buffer. An equal volume of Luciferin Detection Reagent was added, and the luminescent signal was read after 15 minutes on a GloMax® 96 Microplate Luminometer. The background luminescence (no GSH) was subtracted from the signal to calculate the net luminescence. None of the tested reducing agents interfered with the GSH-Glo™ Assay chemistry, demonstrating the specificity of the proluminogenic Luciferin-NT substrate.

with the thiol group. Therefore, measuring GSH levels is important for assessing toxicological responses that can promote oxidative stress and lead to apoptosis and cell death (4).

Detect GSH Concentration Using a Two-Step, Enzyme-Driven Assay

The GSH-Glo™ Assay[a–c] is a luminescence-based assay to detect and quantify GSH in cultured cells and other biological samples. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of glutathione (Figure 1) and catalyzed by glutathione S-transferase (GST). The luminescent signal, which is generated in a coupled reaction with firefly luciferase, is proportional to the amount of reduced glutathione present in the sample. The luminescent signal reaches steady state in 15 minutes and is maintained for several hours with minimal loss (Figure 2). The two-step, enzyme-driven assay generates a stable luminescent signal and is simple, fast and easily adaptable to 96- and 384-well plates. A GSH solution is included in the kit so that a standard curve can be generated, allowing researchers to convert luminescent signal from their samples to a GSH concentration.

Use Less Starting Material and Reduce Compound Interference

When compared to colorimetric and fluorescent assays, the GSH-Glo™ Assay is significantly more sensitive and can detect less than 3 nM GSH, requiring less starting material. The GSH-Glo™ Assay can easily detect GSH from less than 10 mg of tissue or fewer than 300 cells/well in a 384-well format (Figure 3). The proluminogenic substrate is specific for glutathione and is not reactive with other reducing compounds like dithiothreitol, β-mercaptoethanol or cysteine (Figure 4). Using luminescence to monitor GSH levels eliminates most signal interference from test compounds.

In addition to being more sensitive than standard fluorescent GSH assays, results can be obtained in less than one hour after preparing lysate. There is no need to remove protein from samples, eliminating the time-consuming centrifugation and transfer steps associated with the standard fluorescent protocols. Table 1 illustrates the simplicity of the GSH-Glo™ Assay protocol compared to a standard fluorescent assay scheme.

Detect Glutathione in Treated Cells

We have used the GSH-Glo™ Assay to measure changes in GSH levels in HepG2 cells (ATCC, Figure 5) and cryopreserved rat hepatocytes (Xenotech, Figure 6). HepG2 cells were exposed to various levels of BSO L-buthionine-

Detect GSH with a Bioluminescent Assay

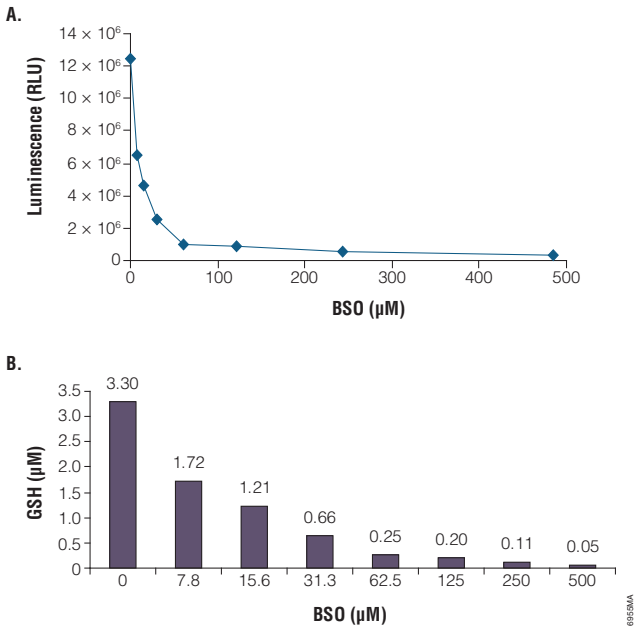

Figure 5. Treatment of HepG2 cells with L-buthionine-sulfoximine (BSO). BSO (Sigma Cat.# B-2515) inhibits GSH synthesis, reducing cellular GSH levels, but it is nontoxic for up to 72 hours (5). HepG2 cells were plated at 5,000 cells/well in a 96-well plate and allowed to attach for approximately 4 hours. Various amounts of BSO were added, and the cells were incubated for 22 hours. The medium was removed from wells, and 100 µl of GSH-Glo™ Reagent was added. A GSH standard curve was generated at the same time as described in Technical Bulletin, #TB369. The GSH-Glo™ Assay reaction was stopped by adding an equal volume of Luciferin Detection Reagent, and luminescence was measured after 15 minutes (**Panel A**). The amount of GSH was calculated based on the standard curve (**Panel B**). The HepG2 cells demonstrated a dramatic dose response to BSO exposure with less than 20% toxicity at the highest dose. Toxicity was determined by cells treated with BSO in parallel but assayed with the CellTiter-Glo® Assay (data not shown).

sulfoximine (Sigma Cat.# B-2515), which inhibits GSH synthesis, thus reducing cellular GSH level. BSO is nontoxic up to 72 hours (4). GSH levels were measured from rat hepatocytes after treatment with either pregnenolone 16α-carbonitrile alone to induce P450 response or with a coupled exposure of pregnenolone 16α-carbonitrile (PCN) and acetaminophen (APAP, 5).

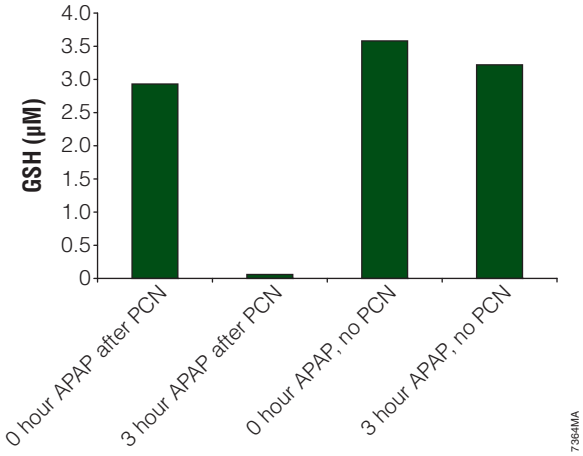

Figure 6. GSH depletion by acetaminophen (APAP) in rat hepatocytes. GSH levels in lysates from adherent cells in 24-well plates were measured with the GSH-Glo® Assay. GSH concentrations were determined by interpolation from a GSH standard curve generated as described in Technical Bulletin #TB369. Exposure to 5 mM APAP for three hours substantially reduced GSH concentrations after treatment for two days with the P450 inducer, 30 µM pregnenolone 16α-carbonitrile (PCN), whereas exposure to APAP or PCN alone caused little difference in GSH levels.

Summary

The GSH-Glo™ Assay provides a luminescent method for detecting GSH in eukaryotic cells that is faster and easier than other commercially available kits. The assay can be completed directly in the multiwell cell-culture plate, which eliminates many cumbersome steps including cell harvesting, washing, lysis, centrifugation and removal of proteins. The luminescent method avoids inherent background fluorescence signals, providing excellent signal-to-background ratios, and the assay format is amenable to high-throughput screening, making this new assay a valuable tool for drug discovery and pharmaceutical applications.

References

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4. Griffith, O.W. and Meister, A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4668–72.
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Protocol

GSH-Glo™ Glutathione Assay Technical Bulletin, #TB369 (www.promega.com/tbs/tb369/tb369.html)

Ordering Information

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

[a]Patent Pending.
[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.
[c]Certain applications of this product may require licenses from others.