Product Manual

OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit

Catalog Number

STA-312 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



Introduction

Oxidative stress occurs when there is an excess of free radicals, or reactive oxygen species (ROS), in the body. Research has shown that excessive ROS accumulation will lead to cellular injury, such as damage to DNA, proteins, and lipid membranes. ROS damage has been implicated in the development of many physiological problems, such as ageing, asthma, arthritis, diabetes, cancer, inflammation, cardiovascular disease, atherosclerosis, Down's Syndrome, and neurodegenerative diseases.

Glutathione is a key intracellular tripeptide thiol composed of glutamic acid, cysteine, and glycine. Glutathione helps protect cells from free radical damage by acting as an antioxidant. Within cells, glutathione exists in reduced (GSH) and oxidized (GSSG) states. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form (GSH) while less than 10% exists in the disulfide form (GSSG). The high GSH concentration is because the enzyme that transitions it from its oxidized state (GSSG), glutathione reductase, is constitutively active and inducible upon oxidative stress. An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. Reduced glutathione's thiol group provides reducing equivalents to other unstable ROS, which in turn becomes unstable itself. This unstable GSH readily reacts with another unstable GSH to form a stable GSSG molecule. This reaction is prevalent since glutathione is present in high concentrations. GSSG is subsequently converted to GSH again by the enzyme glutathione reductase.

In addition to its role in oxidative stress, glutathione also helps maintain exogenous antioxidants such as vitamins C and E. Glutathione is involved with the breakdown of peroxides. It has a role in regulating the nitric acid cycle. Glutathione can directly bind many inorganic and organic xenobiotic (foreign chemicals) and carcinogenic compounds, such as the heavy metals mercury and arsenic. It is important to the proper function and maximum effect of the immune system. In addition, glutathione is fundamentally involved with many metabolic and biochemical mechanisms such as protein and prostaglandin synthesis, DNA synthesis and repair, maintenance of disulfie bonds in proteins, enzyme activation and amino acid transport across cell membranes.

Cell Biolabs' OxiSelect™ Total Glutathione Assay Kit is a quantitative assay for measuring the total concentration of glutathione, which encompasses both reduced and oxidized glutathione (GSH and GSSG) from plasma, blood, saliva, urine, tissue extracts, and plant or mammalian cell lysates. The kit employs a simple enzymatic recycling reaction for total glutathione quantification. The kit has a detection sensitivity limit of 8 nM. Each kit provides sufficient reagents to perform up to 100 assays, including standard curve and unknown samples.

Assay Principle

The OxiSelect™ Total Glutathione Assay Kit is a quantitative assay for measuring the total glutathione content within a sample (GSH/GSSG). Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm (Figure 1). The total glutathione content in unknown samples is determined by comparison with the predetermined glutathione standard curve. The rate of chromophore production is proportional to the concentration of glutathione within the sample. The rate can be determined from the absorbance change over time. Metaphosphoric acid is provided to remove interfering proteins or enzymes from samples.



Figure 1. Assay Principle

Related Products

1. STA-305: OxiSelect™ Nitrotyrosine ELISA Kit
2. STA-310: OxiSelect™ Protein Carbonyl ELISA Kit
3. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
4. STA-340: OxiSelect™ Superoxide Dismutase Activity Assay
5. STA-341: OxiSelect™ Catalase Activity Assay Kit
6. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)
7. STA-347: OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Green Fluorescence)
8. STA-345: OxiSelect™ ORAC Activity Assay
9. STA-346: OxiSelect™ HORAC Activity Assay Kit
10. STA-832: OxiSelect™ MDA Competitive ELISA Kit
11. STA-838: OxiSelect™ HNE Adduct Competitive ELISA Kit

Kit Components

Box 1 (shipped at room temperature)

1. Glutathione Reductase (50X) (Part No. 231201): One 50 µL amber tube.
2. Chromogen (15X) (Part No. 231202): One 1 mL amber tube.
3. Assay Buffer (5X) (Part No. 231204): One 50 mL bottle.
4. Metaphosphoric Acid (MPA) (Part No. 231205): One 2 g bottle of crystals.
5. Glutathione Disulfide (GSSG) (Part No. 231206): One 50 µL amber tube of a 1 mM solution.

Box 2 (shipped on blue ice packs)

1. NADPH (50X) (Part No. 231203): One 50 µL amber tube.

Materials Not Supplied

1. 96-well microtiter plate
2. Distilled or deionized water
3. 1X PBS
4. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
5. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
6. Conical tubes and bottles for sample and buffer preparation
7. Centrifuge and/or microfuge
8. Sonicator or tissue homogenizer
9. Multichannel micropipette reservoirs
10. Microplate reader capable of reading 405 nm

Storage

Upon receipt, store the NADPH at -80ºC. Prepare single use aliquots and avoid multiple freeze/thaw cycles. Store the remaining kit components at 4ºC.

Preparation of Reagents

- 1X Assay Buffer: Prepare 1X Assay Buffer by adding 200 mL of deionized water to 50 mL of the 5X Assay Buffer. Mix thoroughly until homogeneous. Use this buffer for preparing kit reagents. Store at 4ºC when not in use.
- 1X Glutathione Reductase: Prepare the 1X Glutathione Reductase by diluting the stock solution 1:50 with 1X Assay Buffer. Vortex the stock tube thoroughly prior to preparing. Prepare only enough for immediate applications (eg. Add 25 µL of Glutathione Reductase stock to 1.225 mL Assay Buffer).
- 1X Chromogen: Prepare the 1X Chromogen just before use. Prepare only enough for immediate applications. Dilute the Chromogen stock 1:15 with 1X Assay Diluent (eg. Add 167 µL of Chromogen stock to 2.333 mL of 1X Assay Buffer. Vortex thoroughly.
- 1X NADPH: Prepare 1X NADPH by diluting the stock solution 1:50 with 1X Assay Buffer. Vortex the stock tube thoroughly prior to preparing. Prepare only enough for immediate applications (eg. Add 25 µL of NADPH stock to 1.225 mL Assay Buffer).
- Metaphosphoric Acid (MPA): Prepare a 5% (w/v) Metaphosphoric Acid (MPA) solution in deionized water. Prepare just before use. Prepare only enough for immediate applications (eg. Add 0.5 g of Metaphosphoric Acid crystals to 10 mL of deionized water). Vortex thoroughly.

  Note: MPA is corrosive and may cause burns. Use caution when handling acidic reagents.

Preparation of Samples

These preparation protocols are intended as a guide for preparing known samples. The user may need to adjust how they treat their sample accordingly. All samples should be assayed immediately or store at -80°C for up to 1-2 months. A trial assay with a representative test sample should be assayed to determine the samples compatibility with the dynamic range of the standard. It is recommended that samples be processed as soon as possible because GSH is rapidly metabolized and will continue

to form various disulfides. The assay can be used on cell culture supernatants and lysates, blood, plasma, urine, saliva, tissue homogenates as well as other biological fluids. All samples should be treated with 5% MPA to remove interfering proteins and enzymes. The MPA treated deproteinated samples improve the stability of GSH. High levels of interfering substances may cause variations in results. Run proper controls as necessary. Always run a standard curve with samples.

Notes:

- *Thiol compounds, such as cysteine, dithiothreitol (DTT), or β-mercaptoethanol can interfere with the assay by competing with GSH for binding to the Chromogen. In addition, N-ethylmaleimide or other thiol alkylating reagents should also be avoided because they will interfere with Glutathione Reductase and GSH.*
- *5% MPA will interfere with the assay. Upon preparing samples, each sample must be diluted 1:10 with 1X Assay Buffer to bring the MPA concentration to 0.5% final. Some samples may need to be diluted more. Make serial dilutions of samples as necessary to obtain a quantifiable change in absorbance readings over time.*
- *A kinetic assay is recommended because it is more precise than an end point assay.*

- Saliva, Plasma or Urine: GSSG in normal resting saliva, plasma and urine is at or below the detection limit for most glutathione assays. Collect sample in a microfuge tube and immediately add 4 volumes of ice-cold 5% MPA. Mix thoroughly and store on ice for 15 minutes. Centrifuge at 12,000 rpm for 10 minutes at 4ºC to remove insoluble particles. Collect the supernatant. Store on ice if assaying immediately or freeze at -80ºC for future use.

- Cell Lysate: Detach adherent cells by trypsinization. Count cells and centrifuge at 500 rpm for 5 minutes at 4ºC. Wash cells with cold 1X PBS. Centrifuge suspension cells at 500 rpm for 5 minutes at 4ºC. Remove supernatant and wash cells with cold 1X PBS. Repeat centrifugation and remove solution. Immediately resuspend the pellet with 200-500 µL ice-cold 5% MPA for a cell concentration of 1-5 x 10^6 cells. Mix thoroughly. Homogenize or sonicate cell suspension and store on ice until use. Transfer the suspension to a microfuge tube and centrifuge at 12,000 rpm for 5 minutes at 4ºC. Collect the supernatant. Store on ice if used immediately or freeze at -80ºC for future use.

- Tissue Lysate: The GSH concentration in most tissue is in the 1-10 mM range. It is recommended that a 10% w/v homogenate be created. Blood can contaminate a tissue sample due to high GSH concentrations. Therefore, perfusion of the tissue with a PBS/heparin is recommended. Remove tissue and wash the tissue thoroughly with cold isotonic saline solution of 1X PBS with 0.16 mg/mL heparin to prevent coagulation. Blot the tissue dry and weigh. Add ice-cold 5% MPA (~1 mL/100 mg tissue) and homogenize using a glass pestle. Centrifuge the homogenate at 12,000 rpm for 15 minutes at 4ºC. Collect the supernatant. Store on ice if used immediately or freeze at -80ºC for future use.

- Erythrocyte Lysate: Add blood sample to a blood collection tube with an anticoagulant such as heparin or sodium citrate. Centrifuge at 3,000 rpm for 15 minutes at 4ºC. Remove and discard any plasma supernatant. Remove the white buffy coat (leukocytes) on the surface of the erythrocytes. Add four volumes of ice-cold 5% MPA to the pellet and resuspend. Mix thoroughly. Store on ice for 10 minutes. Centrifuge the suspension at 12,000 rpm for 10 minutes at 4ºC. Collect the supernatant. Store on ice if used immediately or freeze at -80ºC for future use.

- Whole Blood Lysate: Collect blood in conical tubes containing an anticoagulant such as sodium citrate or heparin. Add four volumes of ice-cold 5% MPA and mix thoroughly. Store on ice for 10 minutes. Centrifuge the suspension at 12,000 rpm for 10 minutes at 4ºC. Collect the supernatant. Store on ice if used immediately or freeze at -80ºC for future use.

Preparation of Standard Curve

1. Prepare a solution of 0.5% MPA in 1X Assay Buffer and mix. Use this solution for preparing the glutathione standards.
2. To prepare the glutathione standards, first perform a 1:1000 dilution of the stock Glutathione Disulfide (GSSG) in 0.5% MPA/Assay Buffer. Use only enough for immediate applications (eg. Add 2 µL of Glutathione Disulfide to 1998 µL 0.5% MPA /Assay Buffer). This solution has a concentration of 1 µM.
3. Use microfuge tubes to prepare a series of standards according to Table 1 below. Prepare standards fresh for each assay performed. Do not store or reuse standard preparations.

Standard Tubes	1 µM GSSG Standard (µL)	0.5% MPA in Assay Buffer (µL)	GSSG (µM)
1	500	500	0.5
2	500 of Tube #1	500	0.25
3	500 of Tube #2	500	0.125
4	500 of Tube #3	500	0.0625
5	500 of Tube #4	500	0.03125
6	500 of Tube #5	500	0.0156
7	500 of Tube #6	500	0.0078
8	0	500	0

Table 1. Preparation of Glutathione Disulfide Standards

Total Glutathione (GSSG/GSH) Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Prepare the glutathione standards simultaneously with the samples so they may be assayed together. Each sample, including unknown and standard, should be assayed in duplicate or triplicate.
2. In a 96-well plate, add 25 µL of the 1X Glutathione Reductase solution to each well to be tested.
3. Add 25 µL of the 1X NADPH solution to each well to be tested.
4. Add 100 µL of the prepared glutathione standards or samples to each well to be tested. Mix thoroughly.
5. Ensure that the plate reader is prepared for a kinetic assay and is set to read at 405 nm.
6. Add 50 µL of the 1X Chromogen and mix briefly. Immediately begin recording the absorbance at 405 nm at 1 minute intervals for 10 minutes. If using all the wells within the plate at one time, then it may be necessary to record the absorbance at 2 minute intervals.
7. Calculate the concentration of standards and samples. See Calculation of Results below.

Calculation of Results

1. First, determine the average of the replicate absorbance readings for each GSSG standard, sample, and negative control for every time point taken.
2. Graph the average of each standard, sample, and background absorbance at 405nm against incubation time. Determine the slope for each value from the linear portion of each curve. See Figure 2 under Example of Results below.
3. Next, subtract the background slope from the slope of the standards and samples.
4. Plot the net slopes of the GSSG standards against the micromolar concentration of GSSG. See Figure 3 under Example of Results below.
5. Compare the net slopes of the samples with the standard curve from Figure 3 and determine the micromolar concentration of GSSG for each sample. This is equivalent to the total glutathione content.

Example of Results

The following figures demonstrate typical Total Glutathione Assay results. One should use the data below for reference only. This data should not be used to interpret actual results.

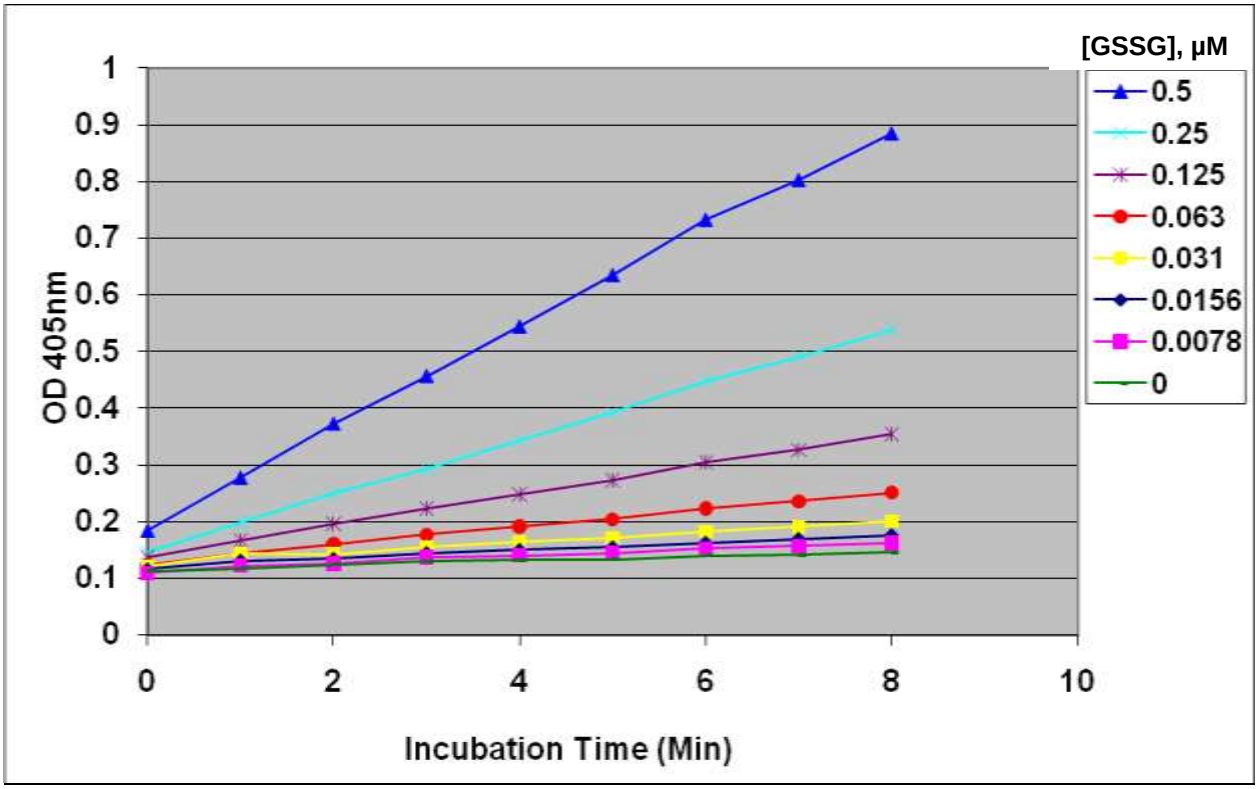


Figure 2. GSSG Standard Curve (OD 405nm versus incubation time as a function of GSSG concentration).

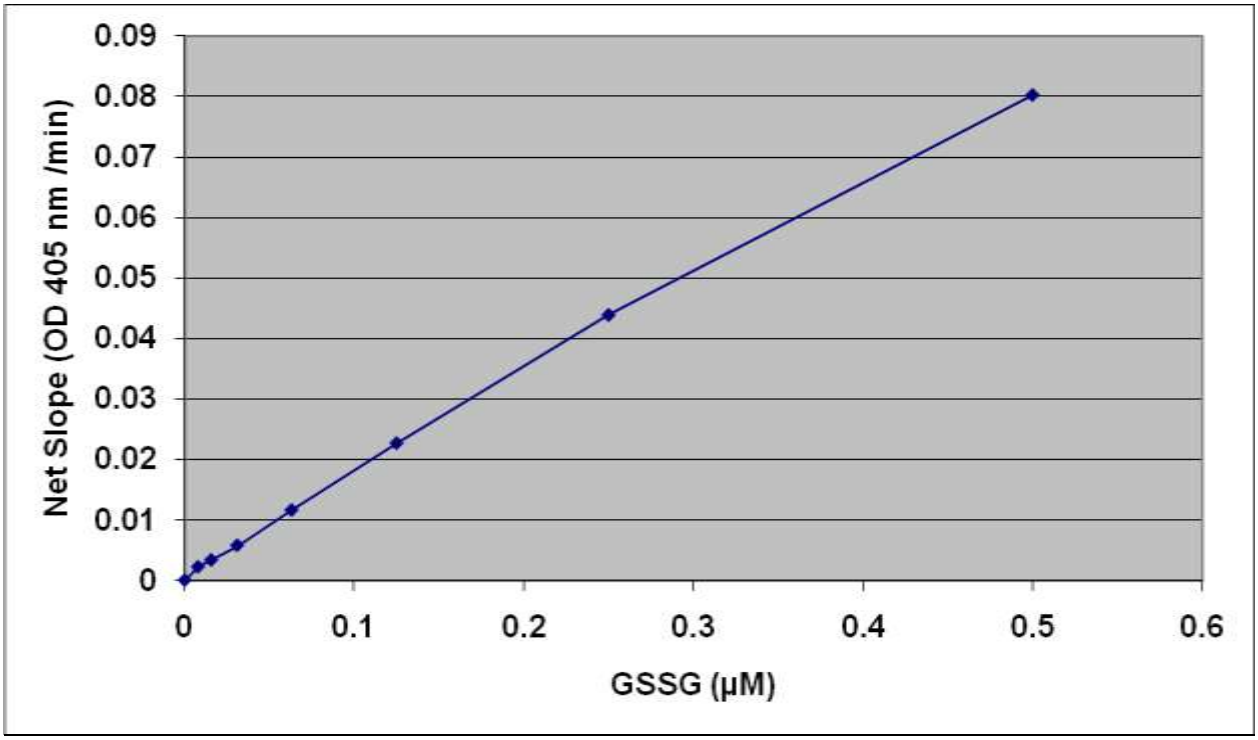


Figure 3: GSSG Standard Curve (Net slope versus GSSG concentration).

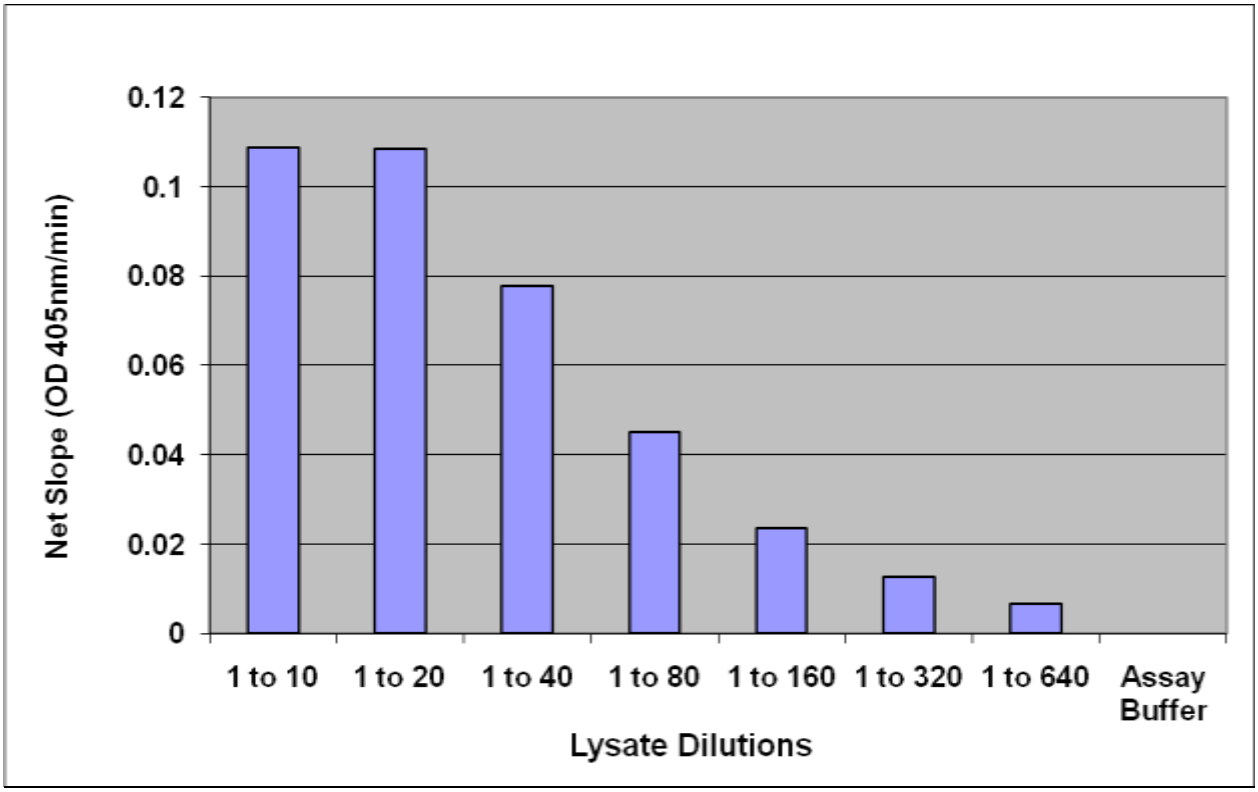


Figure 4. Total Glutathione Content in NIH 3T3 Cell Lysate. Samples were prepared according to the instructions under Sample Preparations above.

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