MANUAL

Total Glutathione (GSSG/GSH) Assay Kit

Catalog #: MA-GSH

ISO 13485:2016

Introduction

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 red blood cells, the reduced form of glutathione is vital in maintaining hemoglobin in a reduced state and hence protecting the cells from oxidative damage. Glutathione is involved in the detoxification of hydrogen peroxide through glutathione oxidase. Low levels of glutathione are found in deficiencies of key enzymes involved in glutathione metabolism, such as glucose-6-phosphate dehydrogenase, glutathione synthase, and glutathione reductase.

Total Glutathione (GSSG/GSH) Assay Kit provides a simple, reproducible, and sensitive tool for measuring total glutathione (both GSH and GSSG) concentration in plasma, serum, cell lysates, urine, and other biological liquid samples. This assay employs an enzymatic recycling method that combines multiple steps. Initially, glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the Colorimetric Probe reacts with the thiol group of GSH to produce a colored compound that absorbs at 405 nm. The optical density measured at 405 nm is directly proportional to the concentration of total glutathione present in the sample.

Storage

The entire kit should be stored below -20 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate	A 96-well (12 strips x 8 wells) plate	RT
Assay Buffer	20 ml	2–8 °C, 3 months
GSSG Standard*	1 vial (Lyophilized)	2–8 °C, 1 week
Glutathione Reductase	1 vial	2–8 °C, 1 week
Cofactor	2 vials (Lyophilized)	Do not store and reuse.
Colorimetric Probe	2 vials (Lyophilized)	Do not store and reuse.

**GSSG is provided as a standard instead of GSH. Under the assay conditions, GSSG is immediately reduced to GSH.*

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 405 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Multi-channel pipettes to deliver 20 µl to 200 µl volumes
4. Tubes to prepare sample dilutions
5. Ultrapure water
6. 50 ml conical tubes
7. Protocatechuic acid (PCA)
8. Potassium hydroxide (KOH)

Sample Tips and General Considerations

These preparation protocols are intended as a guide for preparing known samples. The user may need to adjust their sample preparation methods accordingly. It is recommended to perform a trial assay with a representative test sample to determine the appropriate dilution factor for the samples. Samples should be processed promptly because GSH is rapidly metabolized and will continue to form various disulfides. All samples should be deproteinized to remove interfering proteins and enzymes before assaying. If the assay cannot be performed on the same day, the sample should still be deproteinized, and then stored at -80°C. When you are ready to test your samples, thaw them on ice. Freezing and thawing might affect sample stability, potentially resulting in lower-than-expected readings.

NOTE:

- Thiol compounds, such as cysteine, dithiothreitol (DTT), or β-mercaptoethanol can interfere with the assay by competing with GSH for binding to the Colorimetric Probe. In addition, N-ethylmaleimide or other thiol alkylating reagents should also be avoided because they will interfere with Glutathione Reductase and GSH.
- GSSG in normal resting saliva, plasma, and urine is at or below the detection limit for most glutathione assays.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, and pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes. Keep on ice. General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/
Perform deproteinization step as described in the section “Deproteinization step” below.

2. Plasma samples:

Collect blood with an anticoagulant such as citrate, EDTA, or oxalate and mix by inversion. Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Perform deproteinization step as described in the section “Deproteinization step” below.

3. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Perform deproteinization step as described in the section “Deproteinization step” below.

4. Urine or other biological fluids samples:

To remove insoluble particles, spin at 10,000 x g for 5 min. Perform deproteinization step as described in the section “Deproteinization step” below.

Deproteinization step:

Prepare samples following the protocol above. Keep your samples on ice. Follow the steps below to deproteinize the samples.

1. Add 1 part of ice cold 4 M PCA to 3 parts of the protein sample and vortex briefly to mix well.

NOTE: high protein concentration samples might need more PCA.

2. Incubate on ice for 5 minutes.

3. Centrifuge samples at 13,000 x g for 2 minutes at 4°C and transfer supernatant to a fresh tube. Measure its volume.

4. Add 1 part of ice-cold 2 M KOH to 3 parts supernatant (for instance, 30 µL of 2 M KOH to 90 µL of sample supernatant) and vortex briefly. This will neutralize the sample and precipitate excess PCA.

5. Check pH using pH paper (test 1 µL of sample). Ensure pH is between 6.5 and 8. If necessary, adjust pH with 0.1 M KOH.

6. Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant. Samples are now deproteinized, neutralized, and PCA has been removed. The samples are now ready to use in the assay

Sample concentration calculation

The deproteinized samples will be diluted from the original concentration.
To calculate the dilution factor of your final sample, simply apply the following formula:

$$\% \text{ original concentration} = \frac{Initial\ sample\ volume}{(initial\ sample\ volume + vol\ PCA + vol\ KOH)} \times 100$$

Reagent Preparation

REAGENT	PREPARATION
Microplate	No preparation
Assay Buffer	
GSSG Standard	See “Standard Preparation” section
Glutathione Reductase	Put on ice to thaw and mix well by pipetting up and down. Do not vortex.
Cofactor	For each vial, reconstitute with 1 ml Assay Buffer and mix well
Colorimetric Probe	For each vial, reconstitute with 1 ml Assay Buffer and mix well. *Reconstitution of this reagent should be done just prior to its addition to the **Colorimetric Solution** (described below)*
Reaction Mix	Add 13 µL Glutathione Reductase and 1 mL reconstituted Cofactor into 1850 µL Assay Buffer to make the **Reaction Mix**. This is enough to assay 50 wells. Prepare the additional vial as needed. The **Reaction Mix** is stable for 1 day at 2-8°C in the dark.
Colorimetric Solution	Add 1 mL reconstituted Colorimetric Probe into 1850 µL Assay Buffer to make the **Colorimetric Solution**. This is enough to assay 50 wells. Prepare the additional vial as needed. *The **Colorimetric Solution** should be used immediately.*

Standard Preparation

To prepare a dilution series of the GSSG Standard in the concentration range of 0 µM – 2 µM (see Table below),

1. Label 8 microtubes #1 through 8 with the following concentrations: 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0 µM.
2. Pipette 294 µL Assay Buffer into labeled tube #1, and 150 µL Assay Buffer into labeled tube #2 through #8.
3. Pipette 200 µL Ultrapure water to GSSG Standard tube provided to make 100 µM GSSG Standard Stock.
4. Pipette 6 µL of 100 µM GSSG Standard Stock into tube#1, mix thoroughly, and quick spin to make a 2 µM GSSG Standard.
5. To make the 1 µM standard, pipette 150 µL of tube #1 into the tube labeled #2. Mix thoroughly and quick spin.
6. Repeat this step with each successive concentration, preparing a dilution series as shown in the Table below. Each time, use 150 µl of the prior concentration until the 0.03125 µM is reached. Mix each tube thoroughly before the next transfer. Standards should be prepared fresh and used immediately.

Labeled Tubes	GSSG Standard (µL)	Assay Buffer (µL)	GSSG Conc. (µM)	Equivalent GSH Conc. (µM)*
1	6 µL of 100 µM GSSG Standard Stock	294 µL	2	4
2	150 µL of Tube #1	150 µL	1	2
3	150 µL of Tube #2	150 µL	0.5	1
4	150 µL of Tube #3	150 µL	0.25	0.5
5	150 µL of Tube #4	150 µL	0.125	0.25
6	150 µL of Tube #5	150 µL	0.0625	0.125
7	150 µL of Tube #6	150 µL	0.03125	0.0625
8	0µL	150 µL	0	0

Under the assay conditions, GSSG is reduced to produce 2 mole equivalents of GSH.

Assay Procedure

Each GSSG Standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.
GSSG is provided as a standard instead of GSH. Under the assay conditions, GSSG is immediately reduced to GSH.

1. Add 50 µL of the prepared GSSG Standards or unknown samples to the 96-well microtiter plate.
2. Add 50 µL of Reaction Mix to each well. Mix thoroughly.
3. Ensure that the plate reader is prepared for a kinetic assay and is set to read at 405 nm if using the Kinetic method.
4. Add 50 µL of Colorimetric Solution to each well and mix briefly (a multi-channel pipette is strongly recommended at this step).
5. Total glutathione (GSSG/GSH) concentration of the samples can be determined by the End-point method or the Kinetic method.
   End-point method: Incubate at room temperature in the dark for 5 minutes. Record the absorbance at 405nm.
   Kinetic method: After addition of the Colorimetric Solution, immediately begin recording the absorbance at 405 nm at 1-minute intervals for 5 minutes.
   NOTE: A kinetic assay is recommended because it is more precise than an end-point assay although each method is suitable.

Calculation of Results

End point method:

Subtract the blanks

Average the absorbance value of the blank wells (Standard 0 µM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbances.

Plotting the standard curves

Make a plot of corrected absorbance at 405nm as a function of GSSG concentration.

Determination of sample total glutathione (GSSG/GSH) concentration

GSSG (µM) = $\frac{OD_{Sample}-OD_{Blank}}{Slope_{End}} \times DF$

Total Glutathione (GSSG/GSH) (µM) = 2 × GSSG (µM)

OD_{Sample} = Optical density (OD) reading of the Sample

OD_{Blank} = Optical density (OD) reading of the Blank (Standard 0 µM)

$Slope_{End}$ is from the plot of GSSG concentration vs. Absorbance (as shown in Figure 1 below).

DF = Sample Dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated total glutathione (GSSG/GSH) concentration of the sample is higher than 4 µM, dilute the sample in Assay Buffer and repeat the assay.

Kinetic method:

1. First, determine the average of the replicate absorbance readings for each GSSG standard and samples 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 curve (See Figure 2). This is called i-slope.
3. Next, subtract the background (Standard 0 µM) i-slope from the i-slope of the standards and samples.
4. Plot the i-slopes of the GSSG standards against the concentration of GSSG (See Figure 3). The slope of this curve is called $Slope_{Kinetic}$.
5. Compare the i-slopes of the samples with the standard curve from Figure 3 and determine the concentration of GSSG for each sample.

GSSG (µM) = $\frac{(i\_slope)_{Sample}-(i\_slope)_{Blank}}{Slope_{Kinetic}} \times DF$

Total Glutathione (GSSG/GSH) (µM) = 2 × GSSG (µM)

$(i\_slope)_{Sample}$ = Slope of the sample absorbance at 405nm against incubation time

$(i\_slope)_{Blank}$ = Slope of the Blank (Standard 0 µM) absorbance at 405nm against incubation time

$Slope_{Kinetic}$ is from the plot of GSSG concentration vs. i-slope (as shown in Figure 3 below).

DF = Sample Dilution factor (DF = 1 for undiluted Samples)

Note: If the calculated total glutathione (GSSG/GSH) concentration of the sample is higher than 4 µM, dilute the sample in Assay Buffer and repeat the assay.

A. Typical Data

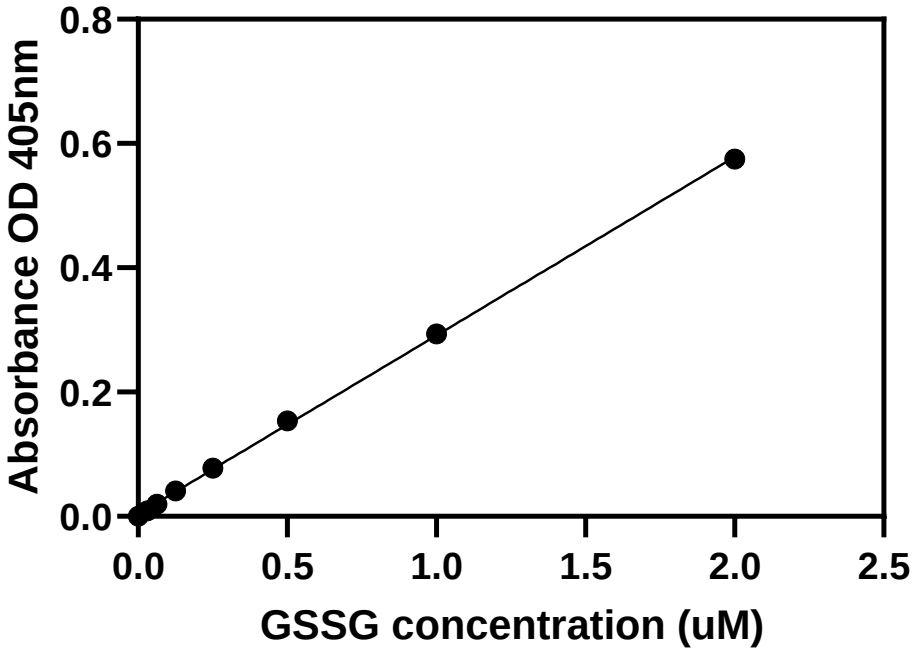

Figure 1. (**End-point method**) Plot of absorbance versus GSSG standard concentration. These standard curves are for demonstration only. A standard curve must be run with each assay.

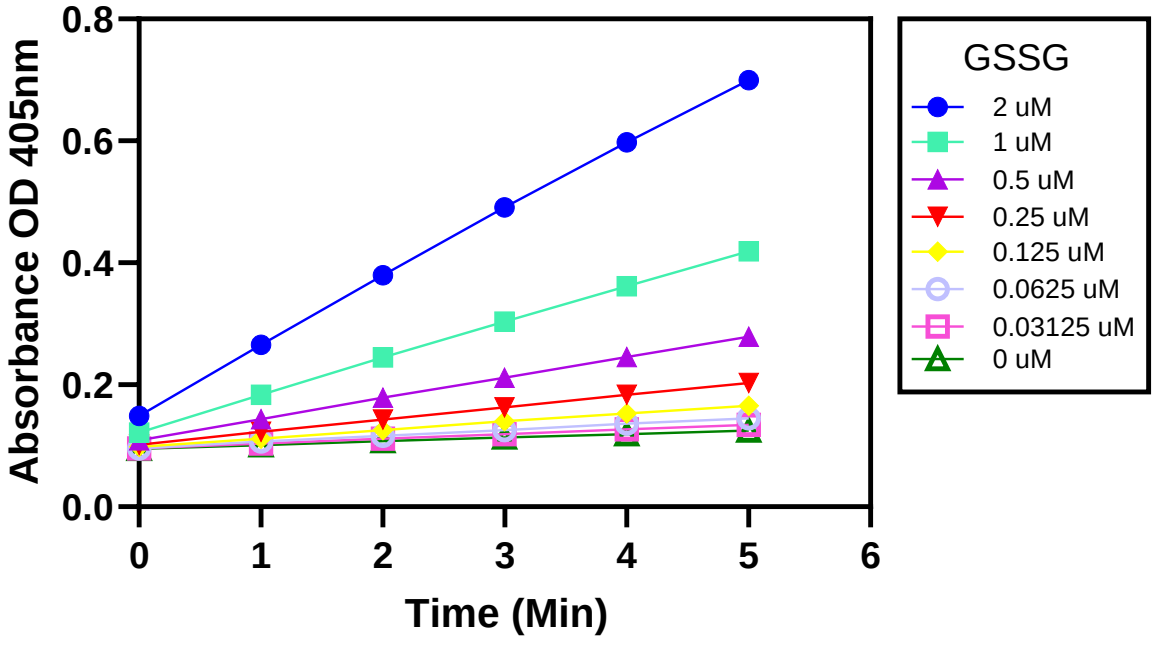

Figure 2. (**Kinetic method**) Plot of absorbance versus time for each GSSG standard.

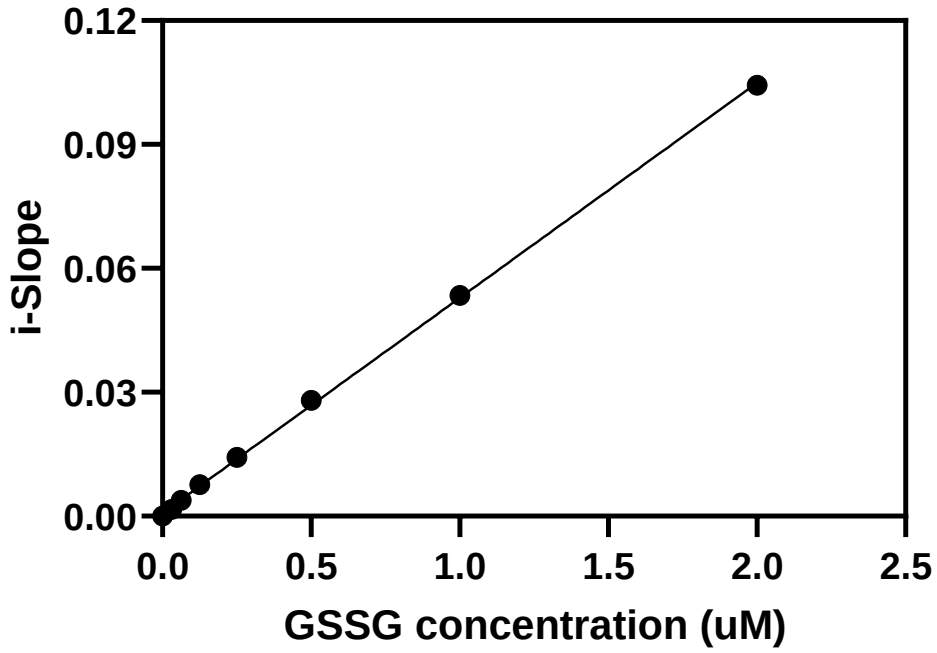

Figure 3. (**Kinetic method**) Plot of i-slope (from Fig. 2) versus GSSG standard concentration. These standard curves are for demonstration only. A standard curve must be run with each assay.

B. Sensitivity

The minimum detectable concentration of GSSG is 8 nM.
Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank.

C. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 2.6%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 3.4%.

This product is for research use only.