KAMIYA BIOMEDICAL COMPANY

# Glutathione Reductase Assay

**For the quantitative determination of glutathione reductase activity in plasma, erythrocytes, tissues and cell lysates**

Cat. No. KT-949

**For Research Use Only. Not for use in diagnostic procedures.**

# Product Information

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## INTRODUCTION

Glutathione reductase is a homodimeric enzyme that is a member of the flavoprotein disulfide oxidoreductases. It has an indirect impact in the prevention of oxidative damage in cells by helping to maintain intracellular reduced glutathione (GSH). Thus, measuring the activity of the enzyme is an indicator of oxidative stress. It is a ubiquitous enzyme that catalyzes the NADPH dependent reduction reaction of oxidized glutathione (GSSG) to reduced glutathione (GSH). Oxidized glutathione is reduced through a multi-step reaction in which glutathione reductase is reduced by NADPH, which in turn reacts with a GSSG molecule. This creates a disulfide interchange reaction that creates two GSH molecules and restores glutathione reductase to its oxidized form. Regenerated GSH is available to detoxify hydrogen peroxide. Maintenance of GSH is vital in oxidation-reduction processes as well as detoxification of hydrogen peroxide and organic peroxides brought on by inflammation in cells.

Our Glutathione Reductase Assay is a quantitative assay for measuring glutathione reductase activity within plasma, erythrocytes, tissues, and cell lysates. Glutathione reductase activity is defined as 1 unit of enzyme reducing 1 µmole oxidized glutathione (GSSG) per minute at pH 7.6 and 25ºC. The kit employs a simple enzymatic recycling reaction for glutathione quantification where the reduction of a chromagen is correlated to glutathione reductase enzymatic activity. The kit has a detection sensitivity limit of approximately 0.6 mU/mL. Each kit provides sufficient reagents to perform up to 100 assays, including calibration curve and unknown samples.

## PRINCIPLE

The Glutathione Reductase Assay is a quantitative assay for measuring the glutathione reductase activity within a sample. 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 glutathione reductase content in unknown samples is determined by comparison with the predetermined glutathione reductase calibration curve. The rate of chromophore production is proportional to the concentration of glutathione reductase activity within the sample. The rate can be determined from the absorbance change over time.


**Figure 1. Assay Principle**

## COMPONENTS

1. Glutathione Reductase Calibrator: One 20 µL amber tube.
2. Glutathione Disulfide (GSSG): One 5 mL bottle of a 10 mM solution.
3. Chromogen: One 0.5 mL amber tube.
4. Assay Buffer (5X): Two 25 mL bottles.
5. NADPH (50X): One 50 µL amber tube.

## MATERIALS REQUIRED BUT NOT SUPPLIED

1. 96-well microtiter plate
2. Distilled or de-ionized water
3. 1X PBS
4. 10 µL to 1,000 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. Ethanol
11. 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.

## REAGENT PREPARATION

• 1X Assay Buffer: Prepare 1X Assay Buffer by adding 200 mL of de-ionized 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.
• Chromogen: Prepare the Chromogen just before use and prepare only enough for immediate applications. Dilute the Chromogen stock 1:15 with 1X Assay Buffer (eg. Add 200 µL of Chromogen stock to 2.8 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).

## SAMPLE PREPARATION

These preparation protocols are intended as a guide for preparing known samples. The user may need to adjust the sample treatment accordingly. All samples should be assayed immediately or stored for up to 1-2 months at -80ºC. A trial assay with a representative test sample should be assayed to determine the samples compatibility with the dynamic range of the calibrator. The assay can be used on cell culture supernatants, plasma, erythrocytes, tissues, and cell lysates. High levels of interfering substances may cause variations in results. Run proper controls as necessary. Always run a calibration 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.
• Make serial dilutions of samples as necessary to obtain a quantifiable change in absorbance readings over time.
• Samples containing reduced glutathione (GSH) should be accounted for by running the sample without Glutathione Disulfide (GSSG) and subtracting these background values from a treated sample. A kinetic assay is recommended because it is more precise than an end point assay.

• Plasma: Add blood sample to a blood collection tube with an anticoagulant such as heparin, EDTA, or sodium citrate. Centrifuge at 3,000 rpm for 10-15 minutes at 4ºC. Remove the upper yellow plasma supernatant layer without disturbing the white buffy coat (leukocytes). Store on ice if assaying immediately or freeze at -80ºC for up to 1-2 months. Dilute the plasma with 1X Assay Buffer before testing.

• Erythrocytes: Add blood sample to a blood collection tube with an anticoagulant such as heparin, EDTA, or sodium citrate. Centrifuge the blood at 3,000 rpm for 10-15 minutes at 4ºC. Remove the white interface buffy coat (leukocytes) and yellow plasma layers and discard. Wash the erythrocytes in cold PBS or saline. Determine the packed cell volume and add 4 cell volumes of cold de-ionized water to lyse the

cells. Centrifuge at 10,000-12,000 rpm for 10 minutes at 4ºC. Collect the supernatant. Store on ice if assaying immediately or freeze at -80ºC for up to 1-2 months. Dilute with 1X Assay Buffer before testing.

• Cell Lysates: Wash adherent cells at 2-6 x $10^6$ cells with 1X PBS. Harvest adherent cells with a rubber policeman, or gentle trypsinization. Centrifuge adherent or suspension cells at 500-1,000 rpm for 5 minutes at 4ºC. Remove supernatant and wash cells in cold PBS. Repeat centrifugation and remove solution. Immediately resuspend the cell pellet with 200-500 µL ice-cold 1X Assay Buffer for a cell number 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 up to 1-2 months. Dilute with 1X Assay Buffer as necessary before testing.

• Tissue Lysate: Perfuse or wash the tissue to remove blood cells and clots with a cold PBS/1 mM EDTA. Homogenize 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 PBS/1 mM EDTA (~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 up to 1-2 months. Dilute with 1X Assay Buffer as necessary before testing.

## CALIBRATOR PREPARATION

1. To prepare glutathione reductase calibrators, first perform a 1:500 dilution of the stock Glutathione Reductase in 1X Assay Buffer. Use only enough for immediate applications (eg. Add 5 µL of Glutathione Reductase to 2,495 µL 1X Assay Buffer). This solution has a concentration of 1 Unit/mL or 1,000 mU/mL.
2. Use microfuge tubes to prepare a series of calibrators according to Table 1 below. Prepare calibrators fresh for each assay performed. Vortex tubes thoroughly when preparing as the glutathione reductase solution is a suspension, and therefore will settle upon standing. Do not store or reuse calibrator preparations.

| Standard Tubes | 1000 mU/mL Glutathione Reductase Standard (µL) | 1X Assay Buffer (µL) | Glutathione Reductase (mU/mL) |
|---|---|---|---|
| 1 | 80 | 920 | 80 |
| 2 | 500 of Tube #1 | 500 | 40 |
| 3 | 500 of Tube #2 | 500 | 20 |
| 4 | 500 of Tube #3 | 500 | 10 |
| 5 | 500 of Tube #4 | 500 | 5 |
| 6 | 500 of Tube #5 | 500 | 2.5 |
| 7 | 500 of Tube #6 | 500 | 1.25 |
| 8 | 0 | 500 | 0 |

**Table 1. Preparation of Glutathione Reductase Calibrators.**

## ASSAY PROCEDURE

1. Prepare and mix all reagents thoroughly before use. Prepare the glutathione reductase calibrators simultaneously with the samples so they may be assayed together. Each sample, including unknown and calibrator, should be assayed in duplicate or triplicate.
2. In a 96-well plate, add 25 µL of the 1X NADPH solution to each well to be tested.
3. Add 100 µL of the prepared glutathione reductase calibrators or samples to each well to be tested.
4. Add 50 µL of the 1X Chromogen and mix briefly.
5. Ensure that the plate reader is prepared for a kinetic assay and is set to read at 405 nm.
6. Add 25 µL of the Glutathione Disulfide (GSSG) solution and mix briefly. Immediately begin recording the absorbance at 405 nm at 1 minute intervals for 10 minutes. The reaction may be run longer if necessary. 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 calibrators and samples. See Calculation of Results on next page.

# CALCULATION OF RESULTS

1. First, determine the average of the replicate absorbance readings for each glutathione reductase calibrator, sample, and negative control for every time point taken.
2. Graph the average of each calibrator, sample, and background absorbance at 405 nm 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 calibrators and samples.
4. Plot the net slopes of the glutathione reductase calibrators against the microunits/mL concentration of glutathione reductase. See Figure 3 under Example of Results below.
5. Compare the net slopes of the samples with the calibration curve from Figure 3 and determine the microunits/mL concentration of glutathione reductase for each sample.

Note: Remember that the calibrators and samples are diluted 1:2 dilution within the final assay reaction protocol.

# EXAMPLE OF RESULTS

The following figures demonstrate typical Glutathione Reductase Assay results at 405 nm. One should use the data below for reference only. This data should not be used to interpret actual results.

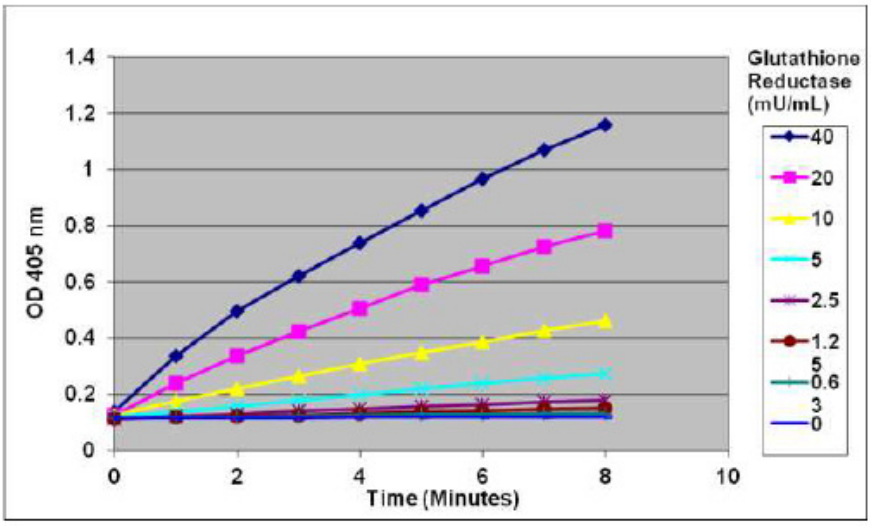


**Figure 2. Glutathione Reductase Calibration Curve** (OD 405 nm versus incubation time as a function of Glutathione Reductase concentration).

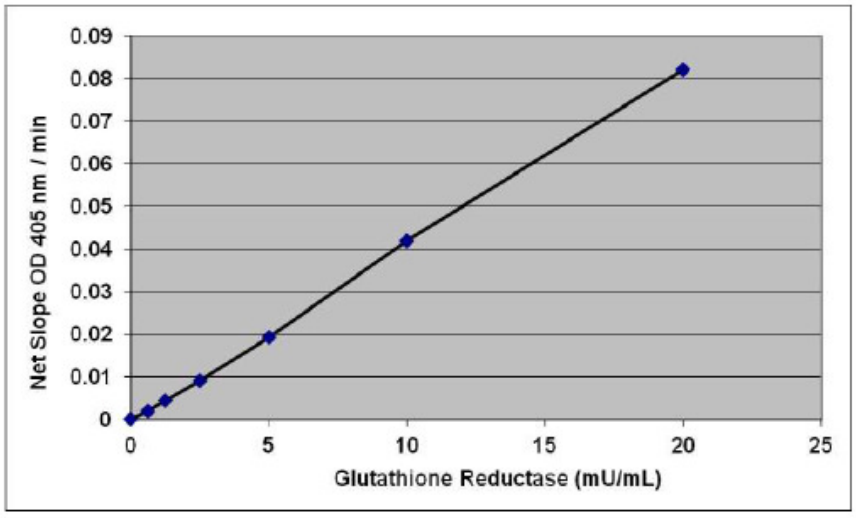


**Figure 3: Glutathione Reductase Calibration Curve** (Net slope versus Glutathione Reductase concentration).

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KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
**www.k-assay.com**