EZ-Glutathione Assay Kit

(GSH/GSSG, Total) Colorimetric

Oxidative Stress Assay Kit

Cat. No. DG-GLU200

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

DoGenBio Co., Ltd.

▪ Product Description

Reduced glutathione (GSH), which has the Free Thiol-Group of Tri-Peptide (γ-glutamyl cysteinyl glycine), converts water and alcohol into hydrogen peroxide and lipid hydroperoxide by glutathione peroxidase (GPx). It is involved in the decomposition reaction and is an antioxidant that plays an important role in human tissue. It is known. In this reaction, GSH is changed into oxidized glutathione (GSSG), and the changed GSSG is reduced to GSH by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH).

When animal cells are exposed to oxidative stress, GSSG accumulates and the GSH/GSSG ratio decreases. The amount of GSSG or GSH/GSSG ratio measured at this time can be used as an accurate indicator to measure oxidative stress and can be effectively used in experiments to monitor the effect of controlling oxidative stress.

The measurement of GSSG is very difficult because very small amounts of GSSG exist in tissues and there is no effective way to prevent the reduction of GSSG to GSH in preparation for measuring GSSG. N-ethylmaleimide (NEM) was first discovered to remove GSH to measure GSSG. However, while NEM reacts with GSH to form a stable complex, it has the disadvantage of inhibiting the enzymatic reaction that occurs with GR (Glutathione Reductase). 2-vinylpyridine (2-VP), which can form GSH without inhibiting GR, was discovered as a substance that improved these shortcomings. However, the 2-VP reaction occurs relatively slowly and has the disadvantage of low solubility in water-soluble solvents.

DoGENBIO Co. Ltd.'s EZ-Glutathione (GSH/GSSG, Total) Assay Kit compensates for the shortcomings of existing materials by using 1-methyl-2-vinylpyridinium trifluoromethanesulfonate1 (M2VP), a thiol-scavenging reagent. This product enables accurate measurement by masking GSH with a short reaction time and not interfering with the reaction with GR.

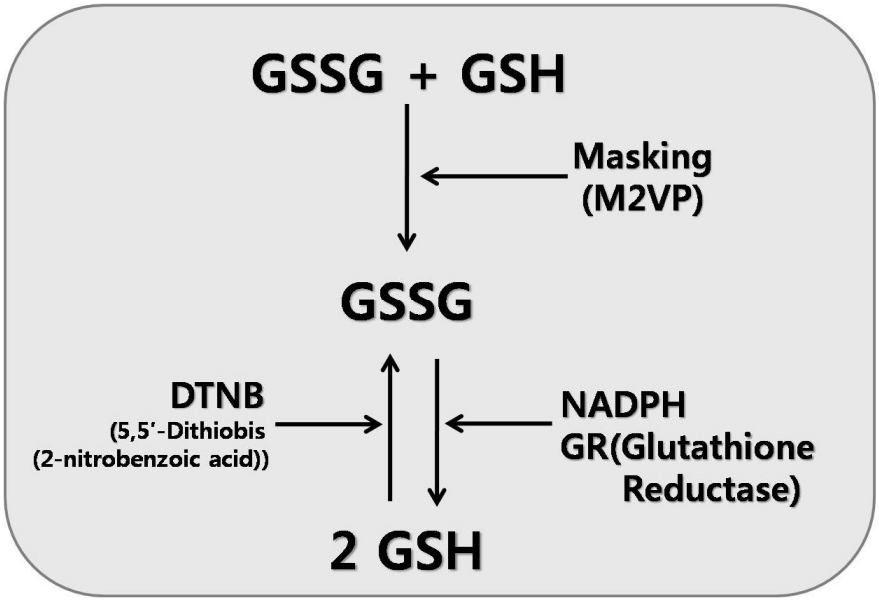

Fig 1. Detection mechanism with EZ-Glutathione assay kit

GSSG is measured by oxidizing GSH to GSSG and then reacting with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB). The EZ-Glutathione (GSH/GSSG, Total) Assay Kit measures the color change that occurs during the reaction, and this color change occurs in proportion to the change in concentration of GSH and GSSG.

▪ Kit Contents and Storage Conditions

Components	200 assay	
Assay Buffer I	1 Bottle	60 ㎖
Assay Buffer II	1 Bottle	60 ㎖
Standard (GSSG Standard)	2 vials	2 ㎖ X 2
Enzyme (GR, Glutathione Reductase)	2 vials	5 ㎖ X 2
NADPH (Lyophilized powder)	2 vials	
Chromogen (DTNB)	2 vials	5 ㎖ X 2
Masking Reagent (M2VP)	2 vials	2 ㎖ X 2

* Store the kit at 4℃ before opening.

▪ Preparation of Solution

Solution	Preparation	Storage
NADPH	Add 5 ml of Assay Buffer II to 1 vial and mix thoroughly. (1 vial is equivalent to approximately 100 tests.)	Dilute immediately before use.
5 % MPA (Metaphosphoric Acid)	Use 1 g of MPA completely dissolved in 20 ml of D.W. (Use by purchasing separately <Sigma, 239275>.)	Store the dissolved solution cold at 4℃ before use. Make a new one and use it during experiments.

▪ Interference

Sample Preparation

** Samples used in experiments are prepared using different processing methods depending on their type and purpose. We recommend that all samples be measured immediately. If not, it can be stored at -80℃ for up to 1 to 2 months. Because GSH reacts rapidly in the sample and continues to produce various disulfides, we recommend processing it as quickly as possible. All samples should be treated with 5% MPA to remove interfering proteins and enzymes and increase stability. Additionally, due to the diversity of experiments, the suggested methods for sample processing may not be accurate. Please be sure to conduct a preliminary experiment.

1. Cell Lysate
   - When cells lyse, GSH is rapidly oxidized. To minimize the oxidation of GSH to GSSG in vitro, cells must be treated with masking reagent (M2VP) before lysis or homogenization.

2. Tissues
   - To measure oxidized GSH in vitro, add M2VP and perform the experiment as quickly as possible.
   - GSSG measurements can be very small. (This may happen if it is washed a lot.)
   - γ-Glutamyltranspeptidase occurs in the metabolism of GSH. It may appear high in membranes such as kidney, pancreas, ciliary body, choroidal network, intestinal epithelium, bile duct epithelial cells, lymphoid cells, and cancer cells.

3. Whole Blood
   - Freezing Step : During the freezing step, red blood cells are lysed to maximize the concentration of GSSG in the sample.
   - Frozen Samples : If GSSG is measured by treating a frozen blood sample with Masking Reagent (M2VP) without pretreatment, it may be inaccurate.

- GSH Linearity : Since GSH exists at a high concentration in whole blood, it must be diluted at least 244 times before use.
- Sample Stability : Glutathione and oxidized glutathione are relatively safe in cells stored at 4°C for 24 hours. Because the stability of elevated GSSG in red blood cells Has not been determined, freeze M2VP-treated blood samples as soon as possible after use.

Limitations

- If thiol-based substances such as cysteine, dithiothreitol (DTT), 2-mercaptoethanol, etc. are present in the sample, reaction interference may occur, making accurate measurement difficult.
- This analysis can be performed on whole blood to which EDTA, an anti-coagulant, has been added.

Assay Performance

The measured curve graph should be straightened. If not, it usually means that the concentration of GSH is high. Dilute the sample and try again. The corrected curve and graph must also be straightened.

▪ General Protocol

1. Sample Preparation

1) Cell Lysate

- Wash the cells separated by trypsin treatment with PBS 2-3 times to completely remove any remaining media and PBS. Suspend cells by adding 200 to 500 µl of cold 5% MPA per approximately 1 to 5 x 10^6 cells. Cell suspensions are sonicated or homogenized on ice or in cold conditions. The sufficiently treated cell suspension is centrifuged at 4°C, 12,000 rpm, for 10 min, and the supernatant is used. If using immediately, use on ice. Otherwise, store immediately at -80°C.

2) Tissues

- GSH in most tissues is present in the range of approximately 1 to 10 mM. Blood components remaining after tissue removal may interfere with accurate measurements due to their high GSH concentration. After tissue extraction, we recommend storing it in PBS/heparin solution. Afterwards, wash several times with PBS containing 0.16 mg/mL of heparin. After completely removing the PBS/heparin solution, the washed tissue is homogenized by adding 1 mL of cold 5% MPA solution per approximately 100 mg of tissue. After homogenization, centrifuge at 4°C, 12,000 rpm, 10 min and use the supernatant. If using immediately, use on ice. Otherwise, store immediately at -80°C.

3) Whole Blood

- A blood sample is collected in a tube containing an anticoagulant such as sodium citrate or

heparin. Dilute by adding approximately 4 times the amount of cold 5% MPA as the collected sample. After reacting on ice for 10 minutes, centrifuge at 4°C, 12,000 rpm for 10 minutes, and use the supernatant. If using immediately, use on ice. Otherwise, store immediately at -80°C.

4) Erythrocyte Lysate

- A blood sample is collected in a tube containing an anticoagulant such as sodium citrate or heparin. Centrifuge at 4°C, 3,000 rpm for 15 min, then remove the supernatant and white buffy coat (leukocyte). Dilute the completely removed sample with approximately 4 times the amount of cold 5% MPA. Afterwards, react on ice for 10 minutes, centrifuge at 4°C, 12,000 rpm, for 10 minutes, and use the supernatant. If using immediately, use on ice. Otherwise, store immediately at -80°C.

5) Saliva, Plasma, Urine

- GSSG present in Saliva, Plasma, and Urine may be difficult to measure with this product. Measurement is not recommended.

2. GSSG Standard Preparation

Prepare the standard by diluting the GSSG Standard solution (10 μM) with Serial Dilution (1:1 dilution) using Assay Buffer I. (The concentration of undiluted stock solution of GSSG Standard is 10 μM.)

1) For accurate measurement, it is recommended to prepare and conduct experiments with more than two replicates each of standards and samples.
2) Standard recommends measurement during each experiment.
3) Blank
   ① cell, tissue, or blood samples : Use mixture of 5 % MPA solution 50 μℓ and Assay Buffer I 700 μℓ
   ② other samples : Use Assay Buffer I
4) When diluting GSSG standard, it is recommended to dilute sufficiently for each concentration and use the required amount.

No	GSSG, μM	GSH, μM
1	0.000	0.000
2	0.156	0.313
3	0.313	0.625
4	0.625	1.250
5	1.250	2.500
6	2.500	5.000
7	5.000	10.000
8	10.000	20.000

Table 1. protocol for GSSG standard curve.

(* In the case of GSH Standard, draw a standard curve with GSSG and double the concentration before use.)

2. GSSG Sample Preparation

1) Add 10 ㎕ of Masking Reagent (M2VP) to the Micro Centrifuge Tube.

2) Carefully add 100 ㎕ of the test sample to the bottom of the tube.

3) Mix gently.

4) Unused samples are stored frozen at -70°C. (Stable for about 30 days.)

5) Incubate the samples to be used for approximately 10 minutes at room temperature.

6) Add 290 ㎕ of cold 5% MPA Solution.

7) Mix by vortexing for 15 to 20 seconds.

8) Centrifuge at 1000 x g or higher for 10 minutes.

9) Mix 50 ㎕ of supernatant and 700 ㎕ of Assay buffer II.

10) Place on ice until use. (Samples were diluted 1/60 times total)

3. GSH Sample Preparation

1) Carefully add 50 ㎕ of sample to the bottom of the Micro Centrifuge Tube.

2) Unused samples are stored frozen at -70°C. (Stable for about 30 days.)

3) For the sample to be used, add 350 ㎕ of cold 5% MPA Solution into the tube.

4) Mix by vortexing for 15 to 20 seconds.

5) Centrifuge at 1000 x g or higher for 10 minutes.

6) Mix 50 ㎕ of supernatant and 3 ㎖ of Assay buffer I.

7) Place on ice until use. (Samples were diluted 488 times total)

4. Assay

1) Place 50 ㎕ each of standard, blank, and sample into a 96 well plate.

2) Add 50 ㎕ of chromogen (DTNB) to each well.

3) Add 50 ㎕ of enzyme (GR) to each well.

4) Incubate at room temperature for 5 minutes.

5) Add 50 ㎕ of NADPH to each well.

6) Measure the change in value over 3 minutes using the kinetic method at 412 nm.

(Measurements are taken at intervals of 30 seconds or less over 3 minutes.)

▪ Calculation

The following steps are required to calculate the concentration of GSH and GSSG and the GSH/GSSG ratio.

1. Rate Determination

When measuring absorbance at 412 nm, the change in value appears linearly depending on the GSH concentration, and the linear function is determined by the following equation.

$$A_{412} = \text{Slope} \times \text{Minutes} + \text{Intercept}$$

The slope of a regression equation is equal to the ratio of the measurements. Because of the DTNB background and the time difference between the time of NADPH addition (start of reaction) and the time of measurement, the intercept in the equation can be ignored.
Select a time period when the change in measured value (slope value) is large.

ex)

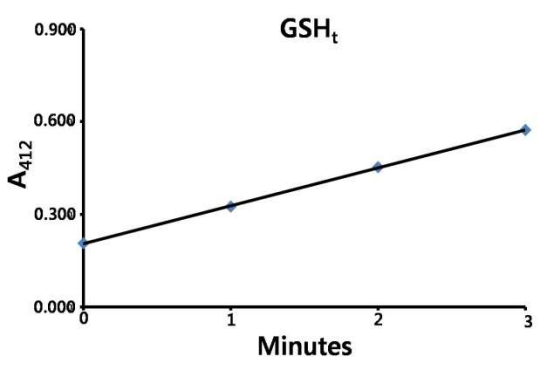

Fig 2. The rate is proportional to the concentration of GSH

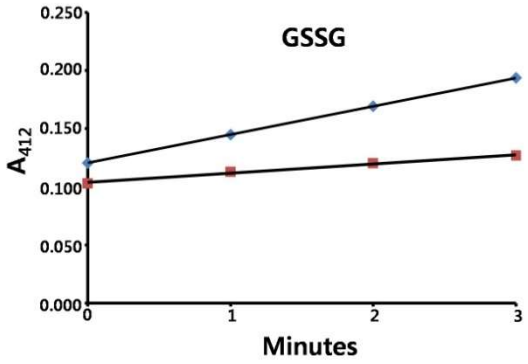

Fig 3. The rate is proportional to the concentration of GSSG (Blue : GSSG, Red : Blank)

- GSH_t : A_{412} = 0.123 x Minutes + 0.205 (r^2 = 0.999)
  GSH_t Sample = 0.123 A_{412}/min
- GSSG : A_{412} = 0.024 x Minutes + 0.120 (r^2 = 1.000)
  GSSG Sample = 0.024 A_{412}/min
- GSH Blank : A_{412} = 0.008 x Minutes + 0.103 (r^2 = 0.999)
  GSH Blank = 0.008 A_{412}/min
  (* This value is calculated by excluding the intercept value.)

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2. Calibration Curve

When measuring the concentration of GSSG in the reactant in which GSHt was measured, the concentration is very low, so it is recommended to select the required range. (At this time, the concentration range of GSSG is expressed by setting it lower than that of GSHt.) Calibration Curve is created by excluding blank values. (Net Rate = Measured Value – Blank Value)

Ex)

GSH (μM)	A_{412}/min	Net Rate
0.00	0.127	0.000
0.25	0.181	0.054
0.50	0.236	0.109
1.00	0.328	0.201
4.00	0.687	0.560
8.00	1.256	1.129

Table 2. A typical 6 point calibration of the EZ-Glutathione Assay Kit.

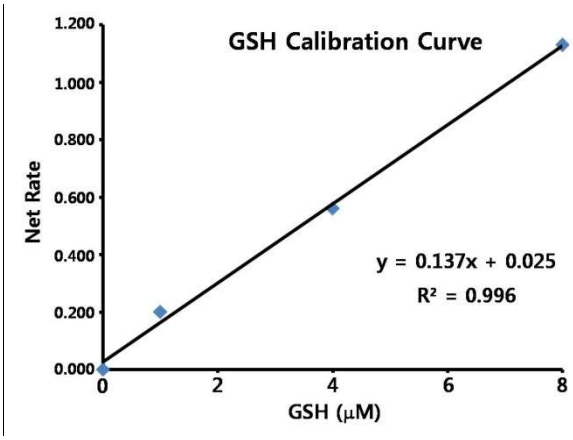

Fig 4. The Calibration Curve of GSH

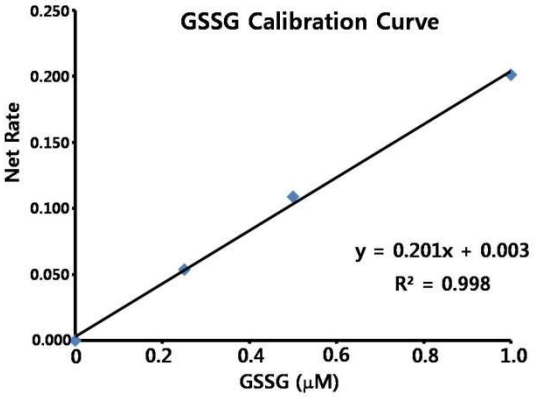

Fig 5. The Calibration Curve of GSSG

3. GSH_t and GSSG Concentrations

The general form of the regression equation to obtain the calibration curve is as follows.
(Net Rate = Measured Value – Blank Value)

$$\text{Net Rate} = \text{Slope} \times \begin{matrix}\text{GSSG} \\ \text{or} \\ \text{GSH}_t\end{matrix} \text{ measure value} + \text{Intercept}$$

To calculate the concentration measured in the calibration curve, modify the equation as follows.

$$\frac{\text{GSSG}}{\text{or}}\ \text{GSH}_t = \frac{\text{Net Rate} - \text{Intercept}}{\text{Slope}} \times \text{Dilution Factor}$$

Ex) In Fig 2, the GSH_t sample can be expressed as 0.496 - 0.127 or 0.369 A_{412}/min, and by substituting this value into the above equation using the equation in Fig 4, which is corrected, the following value can be obtained.

$$GSH_t = \frac{0.369 - 0.025}{0.137} \times 488 = 1225.3\ \mu M$$

In the same way, it can be expressed as 0.194 - 0.127 or 0.067 A_{412}/min in the GSSG sample, and by substituting this value into the above equation using the equation in Fig. 5, which is shown by correcting this value, the following value can be obtained.

$$GSSG = \frac{0.067 - 0.003}{0.201} \times 60 = 19.1\ \mu M$$

4. GSH/GSSG Ratio

GSH/GSSG Ratio is calculated using the following formula.

$$\text{Ratio} = \frac{GSH_t - 2\ GSSG}{GSSG}$$

Ex) Calculating the GSH/GSSG Ratio using the GSH and GSSG values is as follows.

$$\text{Ratio} = \frac{1225.3 - 38.2}{19.1} = 62.15$$

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▪ Related Product

	Products	Catalog No.	Assay
Oxidative Stress Assay Kit	EZ-Superoxide Dismutase (SOD) Assay Kit (Colorimetric)	DG-SOD400	400 Assay
	EZ-Glutathione Assay Kit (Colorimetric)	DG-GLU200	200 Assay
	EZ-Catalase Assay Kit (Fluorometric/Colorimetric)	DG-CAT400	400 Assay
	EZ-Hydrogen peroxide/Peroxidase Assay Kit (Fluorometric/Colorimetric)	DG-PER500	500 Assay
	EZ-Lipid Peroxidation (TBARS) Assay Kit (Colorimetric)	DG-TBA200	200 Assay
	EZ-Total Antioxidant Capacity (TAC) Assay Kit (Colorimetric)	DG-TAC200	200 Assay
	EZ-DPPH Antioxidant Assay Kit (Colorimetric)	DG-DPH400	400 Assay
	EZ-ABTS Antioxidant Assay Kit (Colorimetric)	DG-ABT400	400 Assay
	EZ-Glutathione Peroxidase Assay Kit (Colorimetric)	DG-GPX100	100 Assay
Metabolism Assay Kit	EZ-Lactate Assay Kit (Colorimetric)	DG-LAC100	100 Assay
	EZ-Acetylcholinesterase Assay Kit (Colorimetric)	DG-ACE100	100 Assay
	EZ-Ascorbic Acid Assay Kit (Colorimetric)	DG-ASC100	100 Assay
	EZ-ATP Assay Kit (Fluorometric/Colorimetric)	DG-ATP100	100 Assay
	EZ-Free Fatty Acid Assay Kit (Fluorometric/Colorimetric)	DG-FFA100	100 Assay
	EZ-Free Glycerol Assay Kit (Fluorometric/Colorimetric)	DG-FGC100	100 Assay
	EZ-Glucose Assay Kit (Fluorometric/Colorimetric)	DG-GCS100	100 Assay
	EZ-HDL, LDL/VDL Assay Kit (Fluorometric/Colorimetric)	DG-CHO100	100 Assay
	EZ-Total Cholesterol Assay Kit (Fluorometric/Colorimetric)	DG-TSC100	100 Assay
	EZ-Triglyceride Quantification Assay Kit (Fluorometric/Colorimetric)	DG-TGC100	100 Assay
	EZ-Nitric Oxide Assay kit (Colorimetric)	DG-NO500	500 Assay
	EZ-Total Collagen Assay Kit (Colorimetric)	DG-COL100	100 Assay
	EZ-Ethanol Assay Kit (Colorimetric)	DG-ETH100	100 Assay