

KAMIYA BIOMEDICAL COMPANY

GSSG/GSH Quantification Kit

For the quantification of GSH and GSSG

Cat. No. KT-768

For Research Use Only.

PRODUCT INFORMATION**GSSG/GSH Quantification Kit
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Glutathione (γ -L-glutamyl-L-cysteinylglycine) is a tripeptide compound which exists in a body, and it is involved in antioxidation, drug metabolism, and others as an enzyme substrate of glutathione peroxidase, glutathione S-transferase, thiol transferase, and so on. Glutathione is usually presented as a reduced form (GSH), but GSH is converted into an oxidized form (GSSG) by stimulation of oxidative stress. Therefore, the ratio of GSH and GSSG has been focused as an index of oxidative stress. The GSSG/GSH Quantification Kit contains the reagent for GSH masking. GSH in a sample solution can be removed by adding the Masking reagent. Therefore, GSSG in the sample solution can be selectively determined by measuring the absorption ($\lambda_{\max} = 412 \text{ nm}$) derived from a colorimetric reaction of DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) coupled with the enzymatic recycling system. The quantity of GSH can also be determined by subtracting the amount of GSSG from the total amount of glutathione. The detection ranges of total glutathione and GSSG using in this kit are from 0.5 $\mu\text{mol/L}$ to 50 $\mu\text{mol/L}$ and from 0.5 $\mu\text{mol/L}$ to 25 $\mu\text{mol/L}$, respectively.

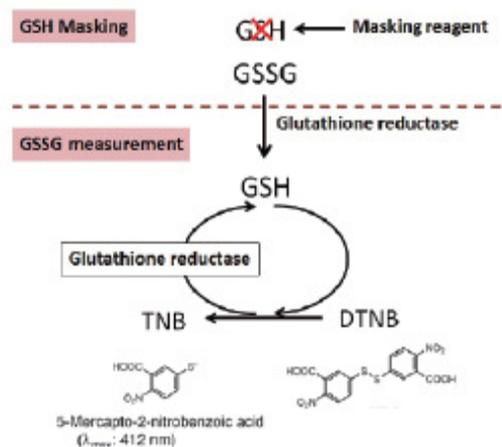


Fig.1 Principle of GSSG/GSH detection

COMPONENTS

• Enzyme Solution	50 μL x 1
• Buffer Solution	60 mL x 1
• GSH Calibrator	x 1
• Masking Reagent	20 μL x 1
• Coenzyme	x 2
• Substrate (DTNB)	x 4
• GSSG Calibrator	x 1

Materials or equipment required but not provided

- Plate reader (405 or 415 nm filter)
- Incubator
- 5-Sulfosalicylic acid (SSA) solution
- 96-well microplate
- 20 μL and 200 μL pipettes, a multi channel pipette
- Ethanol

PROCEDURE**Precautions**

- Use the kit after returning to room temperature.

- Masking reagent is lachrymatory and irritating. Handle the reagent in a chemical hood.
- Triplicate measurement per sample is recommended to obtain accurate data.
- Since the reaction starts immediately after the addition of Enzyme working solution to the well, use a multichannel pipette to avoid the reaction time lag of each well.
- If a concentration range of total glutathione of a sample solution is not known, prepare multiply diluted sample solutions.
- This kit contains glass bottles with an aluminum cap. Please handle carefully.

Preparation of Samples

Tissue (100 mg)

- 1) Homogenize the tissue in 0.5-1.0 mL of 5% SSA.
- 2) Centrifuge the homogenized tissue sample at 8,000 xg for 10 minutes.
- 3) Transfer the supernatant to a new tube and add double-deionized H₂O (ddH₂O) to reduce the SSA concentration to 0.5 % for the assay.

Cells (1×10^7 cells)

- 1) Collect cells by centrifugation at 200 xg for 10 minutes at 4°C. Discard the supernatant.
- 2) Wash the cells with 300 μ L of PBS and centrifuge at 200 xg for 10 minutes at 4°C. Discard the supernatant.
- 3) Add 80 μ L of 10 mmol/L HCl, and lyse cells by freezing and thawing twice.
- 4) Add 20 μ L of 5% SSA and centrifuge at 8,000 xg for 10 minutes.
- 5) Transfer the supernatant to a new tube, and add ddH₂O to reduce the SSA concentration to 0.5% for the assay.

Plasma

- 1) Centrifuge an anticoagulant-treated blood at 1,000 xg for 10 minutes at 4°C.
- 2) Transfer the top plasma layer to a new tube and add the half the volume of 5% SSA equivalent to the tube.
- 3) Centrifuge at 8,000 xg for 10 minutes at 4°C.
- 4) Transfer the supernatant to a new tube and add ddH₂O to reduce the SSA concentration to 0.5 % for the assay.

Erythrocytes

- 1) Centrifuge an anticoagulant-treated blood at 1,000 xg for 10 minutes at 4°C.
- 2) Discard the supernatant and the white buffy coat.
- 3) Add 4 times volume of 5% SSA to the erythrocytes to lysis.
- 4) Centrifuge at 8,000 xg for 10 minutes at 4°C.
- 5) Transfer the supernatant to a new tube, and add ddH₂O to reduce the SSA concentration to 0.5% for the assay.

Preparation of Solutions

- Substrate working solution

Add 1.2 mL of Buffer solution to a Substrate vial, and dissolve.

** Please make sure that all of the Substrate is dissolved with the Buffer solution. If the Substrate does not dissolve completely, use a vortex mixer or a ultra sonic bath.*

- Enzyme working solution

Pipette to mix Enzyme solution, and take 20 μ L to dilute with 4 mL Buffer solution.

** Enzyme solution may be on the wall or an inside of a cap of tube. Please shake down before opening. Do not use a vortex mixer to prepare the working solution.*

- Coenzyme working solution

Add 2.4 mL of ddH₂O to a Coenzyme vial and dissolve.

** Since the Coenzyme is packed under reduced pressure, open after adding ddH₂O with a syringe through a rubber septum.*

- 200 μ mol/L GSH calibrator solution

Add 2.0 mL of 0.5 % SSA solution to a Calibrator GSH vial and dissolve.

** Since the Calibrator GSH is packed under reduced pressure, open after adding 0.5 % SSA solution with a syringe through a rubber septum.*

- 100 μ mol/L GSSG calibrator solution

Add 2.0 mL of 0.5 % SSA solution to the Calibrator GSSG vial and dissolve.

** Since the Calibrator GSSG is packed under reduced pressure, open after adding 0.5 % SSA solution with a syringe through a rubber septum.*

- Masking solution

Add 180 μL of ethanol to a Masking reagent vial and dissolve.

* *Masking reagent is lachrymatory and irritating. Handle the reagent in a chemical hood.*

Storage conditions of each solution:

Store at 4°C : Enzyme working solution, Masking solution

Store at -20°C : GSH calibrator solution, GSSG calibrator solution, Substrate working solution, Coenzyme working solution

*All solutions are stable for 2 months under designated conditions.

General Protocol**1. Sample preparation**

1) Prepare two sets of sample solutions (200 μL each x 2) if both GSH and GSSG have to be determined.

- For GSSG measurement:

Add 200 μL of the sample to microtube, 4 μL of Masking solution, and mix using vortex mixer. (Sample A)

- For total glutathione measurement: Prepare 200 μL sample. (Sample B)

* If a concentration range of total glutathione in a sample solution is not known, prepare multiply diluted sample solutions.

2. Preparation of GSSG calibrator solution

1) Mix 100 μL of 100 $\mu\text{mol/L}$ GSSG calibrator solution and 300 μL of 0.5% SSA solution in a microtube to prepare a 25 $\mu\text{mol/L}$ GSSG calibrator solutions. Prepare the following GSSG calibrator solutions by serial dilution using 0.5% SSA solution: 25.0, 12.5, 6.25, 3.13, 1.57, 0.78 and 0 $\mu\text{mol/L}$.

2) Add 4 μL of Masking solution to all prepared GSSG calibrator solutions, and mix using a vortex mixer.

3. Preparation of GSH calibrator solution

1) Mix 100 μL of 200 $\mu\text{mol/L}$ GSH calibrator solution and 300 μL of 0.5% SSA solution in a microtube to prepare a 50 $\mu\text{mol/L}$ GSH calibrator solution. Prepare the following GSH calibrator solutions by serial dilution using 0.5% SSA solution: 50.0, 25.0, 12.5, 6.25, 3.13, 1.57 and 0 $\mu\text{mol/L}$

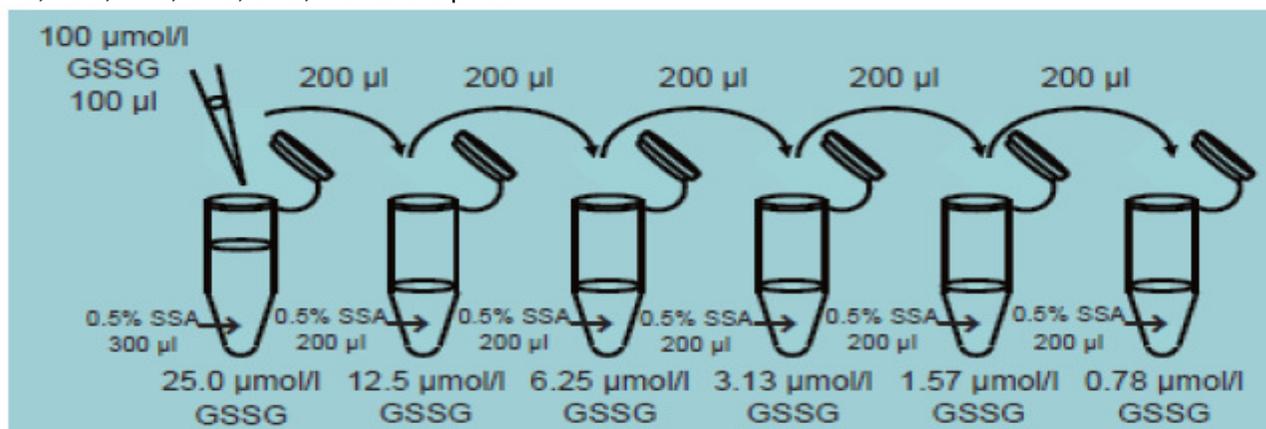


Fig. 2 Preparation of GSSG standard solution

4. Measurement

1) Add 40 μL of GSSG calibrator solution, GSH calibrator solution, Sample A, or Sample B to each well.

* *Triplicate measurement per sample is recommended to obtain accurate data.*

2) Add 120 μL of Buffer solution to each well.

3) Incubate the plate at 37°C for 1 hour.

* *Use a well cap for the microplate to prevent evaporation of the solution during the incubation.*

4) Add 20 μL of Substrate working solution to each well.

5) Add 20 μL of Coenzyme working solution and then 20 μL of Enzyme working solution to each well.

* *Since the reaction starts immediately after the addition of Enzyme working solution, use a multichannel pipette to avoid the reaction time lag of each well.*

6) Incubate at 37°C for 10 minutes.

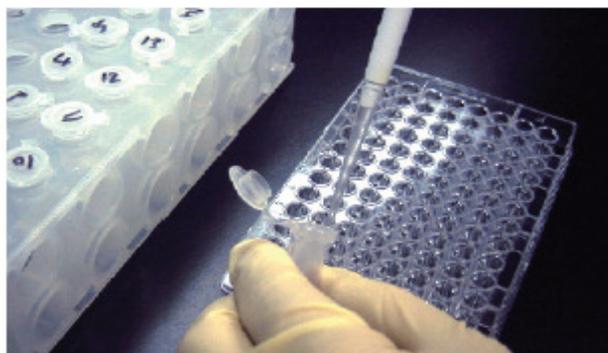
* *If you choose a kinetics method, select "Kinetic" mode.*

* Since the O.D. increases linearly over 10 minutes after the start of reaction, glutathione concentration can be determined by using kinetic method or pseudo-end point method. (measurement of the O.D. at certain time points between 5 and 10 minutes without a stopping reaction)

- 7) Read the absorbance at 405 or 415 nm using a microplate reader.
- 8) Determine the concentration of GSSG in the sample (Sample A) using a GSSG calibration curve.
- 9) Determine the concentration of total glutathione (GSH + GSSG) in the sample (Sample B) using a GSH calibration curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 $\mu\text{mol/l}$ GSSG			Sample A ②			0 $\mu\text{mol/l}$ GSH			Sample B ②		
B	0.78 $\mu\text{mol/l}$ GSSG			Sample A ③			1.57 $\mu\text{mol/l}$ GSH			Sample B ③		
C	1.57 $\mu\text{mol/l}$ GSSG			Sample A ④			3.13 $\mu\text{mol/l}$ GSH			Sample B ④		
D	3.13 $\mu\text{mol/l}$ GSSG			Sample A ⑤			6.25 $\mu\text{mol/l}$ GSH			Sample B ⑤		
E	6.25 $\mu\text{mol/l}$ GSSG			Sample A ⑥			12.5 $\mu\text{mol/l}$ GSH			Sample B ⑥		
F	12.5 $\mu\text{mol/l}$ GSSG			Sample A ⑦			25.0 $\mu\text{mol/l}$ GSH			Sample B ⑦		
G	25.0 $\mu\text{mol/l}$ GSSG			Sample A ⑧			50.0 $\mu\text{mol/l}$ GSH			Sample B ⑧		
H	Sample A ①			Sample A ⑨			Sample B ①			Sample B ⑨		

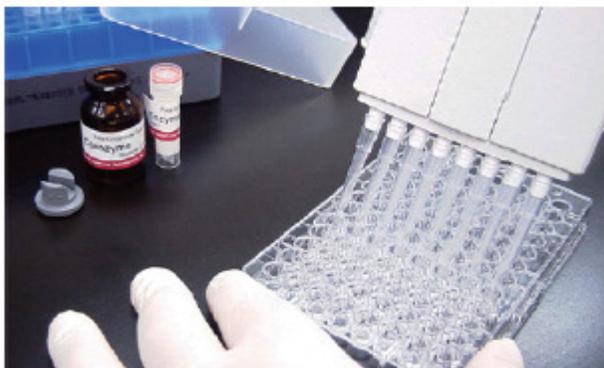
Fig. 3 An example of plate arrangement (n=3)
 (Sample A: Measurement for GSSG, Sample B: Measurement for total glutathione)



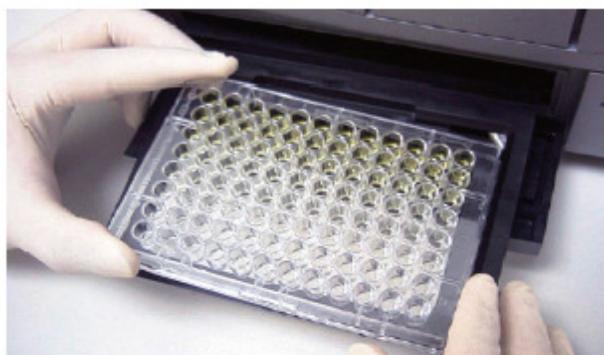
1) Add 40 μL of GSSG calibrator solution, GSH calibrator solution, Sample A, or Sample B to each well.



3) Incubate the plate at 37°C for 1 hour.



4)-5) Add 20 μL of Substrate working solution, 20 μL of Coenzyme working solution and 20 μL of Enzyme working solution to each well.



7) Read the absorbance at 405 or 415 nm using a microplate reader.

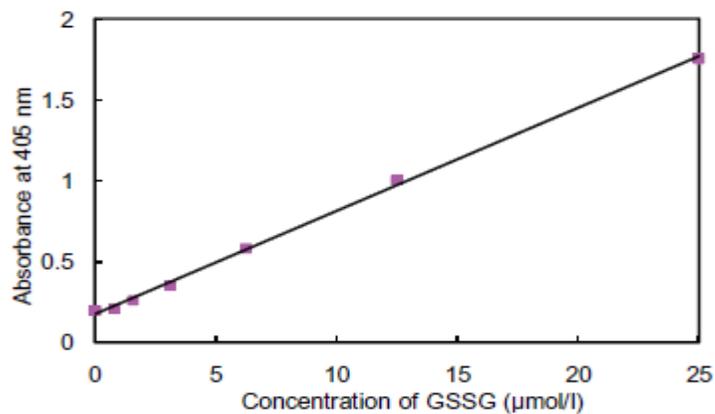


Fig.4 Typical calibration curve of GSSG

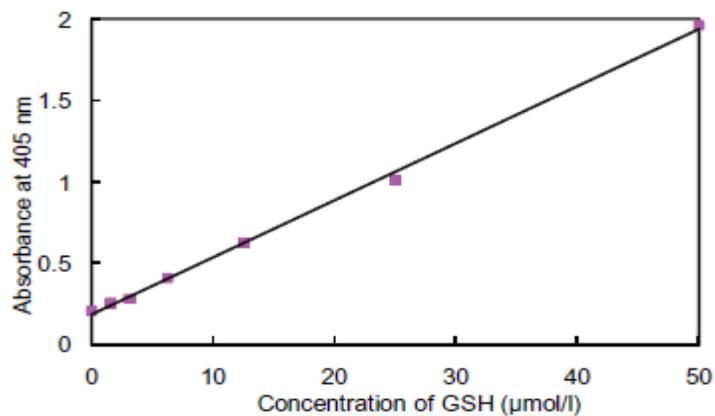


Fig.5 Typical calibration curve of GSH

The concentration of glutathione can be determined by either Kinetic method or Pseudo-end point method.

Pseudo-end point method: Glutathione (GSH, GSSG) = $(O.D._{\text{sample}} - O.D._{\text{blank}}) / \text{slope}^a$

Kinetic method: Glutathione (GSH, GSSG) = $(\text{Slope}_{\text{sample}}^b - \text{Slope}_{\text{blank}}^b) / \text{slope}^a$

a) The slope of the calibration curve prepared by the pseudo-end point or the kinetic method

b) The slope of the kinetic reaction.

10) GSH concentration is calculated using the following equation from the concentrations of total glutathione (GSH + GSSG) and GSSG.

GSH = total glutathione (GSH + GSSG) – GSSG x 2

* *The concentration of glutathione in the sample solution can be calculated from the dilution ratio.*

INTERFERENCE

Reducing agents such as ascorbic acid, β -mercaptoethanol, dithiothreitol (DTT) and cysteine, or thiol reactive compounds such as maleimide compounds, interfere with the glutathione assay. Therefore, these materials should be avoided during the sample preparation.

STORAGE

Store at 4 °C. Use the kit after returning to room temperature.

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