# GSSG/GSH Quantification Kit

# Technical Manual

**Technical Manual (Japanese version) is available at http://www.dojindo.co.jp/manual/g257.pdf**

## General Information

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 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 µmol/l to 50 µmol/l and from 0.5 µmol/l to 25 µmol/l, respectively.

## Principle

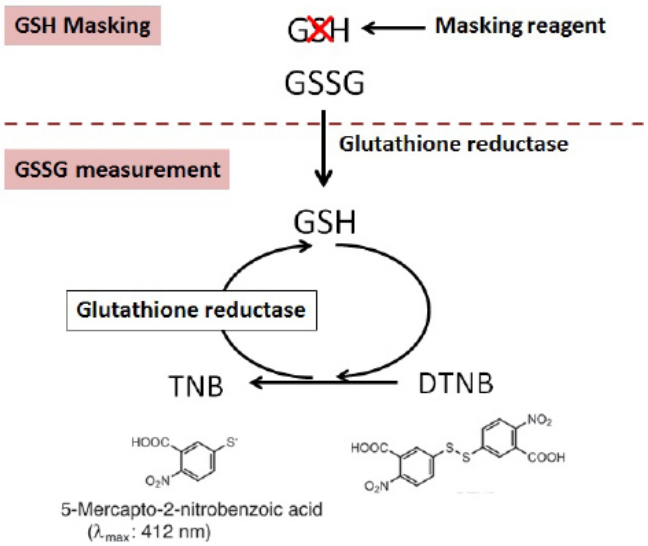



**Fig.1 Principle of GSSG/GSH detection**

## Kit Contents

| | | | |
|---|---|---|---|
| - Enzyme solution | 50 µl x 1 | - Coenzyme | x 2 |
| - Buffer solution | 60 ml x 1 | - Substrate (DTNB) | x 4 |
| - Standard GSH | x 1 | - Standard GSSG | x 1 |
| - Masking reagent | 20 µl x 1 | | |

## Storage Condition

Store at 0-5°C. Use the kit after returning to room temperature.

## Required Equipment and Materials

- 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

## Precaution

- 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.

## Example for Sample Preparation

**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 x*g* for 10 minutes.
3) Transfer the supernatant to a new tube and add double-deionized $H_2O$ (dd$H_2O$) to reduce the SSA concentration to 0.5% for the assay.

**Cells (1x10$^7$ cells)**
1) Collect cells by centrifugation at 200 x*g* for 10 minutes at 4°C. Discard the supernatant.
2) Wash the cells with 300 µl of PBS and centrifuge at 200 x*g* for 10 minutes at 4°C. Discard the supernatant.
3) Add 80 µl of 10 mmol/l HCl, and lyse cells by freezing and thawing twice.
4) Add 20 µl of 5% SSA and centrifuge at 8,000 x*g* for 10 minutes.
5) Transfer the supernatant to a new tube, and add dd$H_2O$ to reduce the SSA concentration to 0.5% for the assay.

**Plasma**

1) Centrifuge an anticoagulant-treated blood at 1,000 x*g* 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 x*g* for 10 minutes at 4°C.
4) Transfer the supernatant to a new tube and add $ddH_2O$ to reduce the SSA concentration to 0.5 % for the assay.

**Erythrocytes**

1) Centrifuge an anticoagulant-treated blood at 1,000 x*g* 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 x*g* for 10 minutes at 4°C.
5) Transfer the supernatant to a new tube, and add $ddH_2O$ to reduce the SSA concentration to 0.5% for the assay.

## *Preparation of Solution*

**- 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 µl to dilute with 4 ml of 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 $ddH_2O$ to a Coenzyme vial and dissolve.

** Since the Coenzyme is packed under reduced pressure, open after adding $ddH_2O$ with a syringe through a rubber septum.*

**- 200 µmol/l GSH standard solution**

Add 2.0 ml of 0.5 % SSA solution to a Standard GSH vial and dissolve.

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

**- 100 µmol/l GSSG standard solution**

Add 2.0 ml of 0.5 % SSA solution to the Standard GSSG vial and dissolve.

** Since the Standard 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 mix by pipetting.

** 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 standard solution, GSSG standard 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 with a 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 standard solution**

1) Mix 100 µl of 100 µmol/l GSSG standard solution and 300 µl of 0.5% SSA solution in a microtube to prepare a 25 µmol/l GSSG standard solutions. Prepare the following GSSG standard solutions by serial dilution using 0.5% SSA solution: 25.0, 12.5, 6.25, 3.13, 1.57, 0.78 and 0 µmol/l.
2) Add 4 µl of Masking solution to all prepared GSSG standard solutions, and mix with a vortex mixer.

**3. Preparation of GSH standard solution**

1) Mix 100 µl of 200 µmol/l GSH standard solution and 300 µl of 0.5% SSA solution in a microtube to prepare a 50 µmol/GSH standard solution. Prepare the following GSH standard solutions by serial dilution using 0.5% SSA solution: 50.0, 25.0, 12.5, 6.25, 3.13, 1.57 and 0 µmol/l.

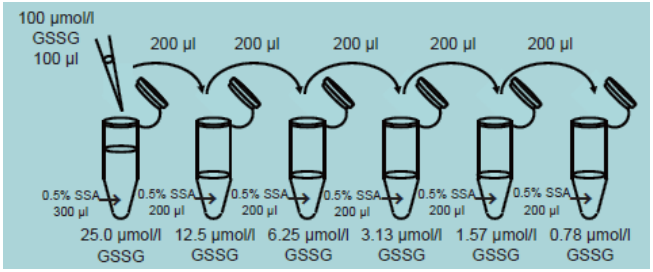



**Fig. 2 Preparation of GSSG standard solution**

## 4. Measurement

1) Add 40 µl of GSSG standard solution, GSH standard 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.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 µmol/l GSSG | | | Sample A ② | | | 0 µmol/l GSH | | | Sample B ② | | |
| B | 0.78 µmol/l GSSG | | | Sample A ③ | | | 1.57µmol/l GSH | | | Sample B ③ | | |
| C | 1.57 µmol/l GSSG | | | Sample A ④ | | | 3.13 µmol/l GSH | | | Sample B ④ | | |
| D | 3.13 µmol/l GSSG | | | Sample A ⑤ | | | 6.25 µmol/l GSH | | | Sample B ⑤ | | |
| E | 6.25 µmol/l GSSG | | | Sample A ⑥ | | | 12.5 µmol/l GSH | | | Sample B ⑥ | | |
| F | 12.5 µmol/l GSSG | | | Sample A ⑦ | | | 25.0 µmol/l GSH | | | Sample B ⑦ | | |
| G | 25.0 µmol/l GSSG | | | Sample A ⑧ | | | 50.0 µmol/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)

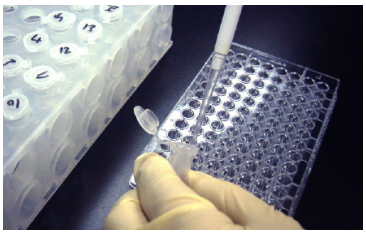
5) Add 20 µl of Coenzyme working solution and then 20 µl of Enzyme working solution to each well.
   ** Since the reaction starts immediately after the addition of Enzyme working solution, use a multichannel pipette to avoid the reaction time lag of each well.*

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)*

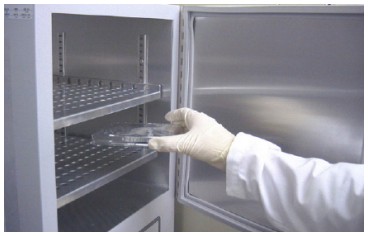
7) Read the absorbance at 405 or 415 nm using a microplate reader.

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) Add 40 µl of GSSG standard solution, GSH standard solution, Sample A, or Sample B to each well.



3) Incubate the plate at 37ºC for 1 hour.

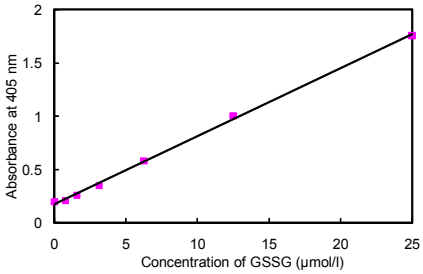
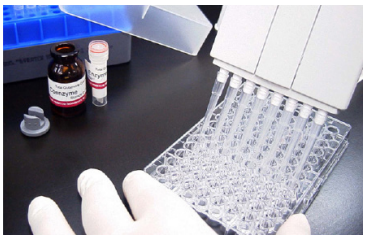
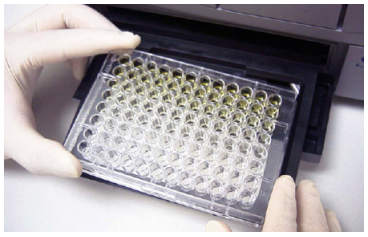



**Fig.4 Typical calibration curve of GSSG**



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.

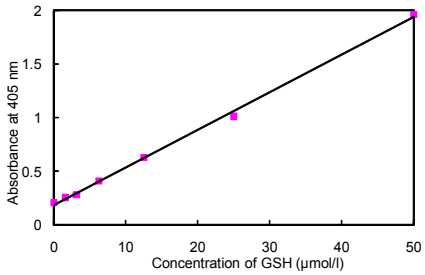



**Fig.5 Typical calibration curve of GSH**

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. $_{sample}$ – O.D. $_{blank}$) / slope [a)]

Kinetic method: Glutathione (GSH, GSSG) = (Slope $_{sample}$ [b)] – Slope $_{blank}$ [b)]) / 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.

*If you need more infomation, please contact Dojindo technical service.*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