GSH and GSSG Assay Kit

Introduction

Glutathione is a small peptide composed of three amino acid residues, with the full name of Glutamyl-cysteinyl-glycine. Since the thiol group (-SH) on cysteine is the active group of glutathione, it is often abbreviated as G-SH or GSH. Glutathione includes two forms: oxidized glutathione (Oxidized glutathione disulfide) and reduced glutathione (Reduced glutathione, commonly referred to as GSH). Oxidized glutathione is formed by the dehydrogenation of two GSH molecules through the thiol group, commonly abbreviated as G-S-S-G or GSSG. Reduced glutathione is the main source of thiol groups in the vast majority of living cells, plays an important role in maintaining the appropriate redox state of thiol groups in proteins, and is a key antioxidant in animal cells. Typically, 90-95% of the Total Glutathione is reduced glutathione.

In the detection of Total Glutathione, first, glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). Subsequently, GSH reacts with the chromogenic substrate DTNB to produce yellow TNB. The amount of Total Glutathione determines the formation amount of yellow TNB, and the amount of Total Glutathione can be obtained by detecting the absorbance at A_{412}. First, use an appropriate reagent to remove GSH from the sample, and then the content of GSSG can be determined using the above reaction principle. Subtract the content of GSSG from the total amount of glutathione (GSSG + GSH) to calculate the content of GSH.

This kit is a simple and easy-to-use detection kit that can separately detect the contents of reduced glutathione GSH and oxidized glutathione GSSG. It can be used to detect the contents of GSH and GSSG in animal tissues, plasma, red blood cells, cultured cells, or other appropriate samples. The detection limit of this kit is 0.5 μM. One kit can perform 100 detections in total, which can determine the Total Glutathione or GSSG content of 100 samples, or can determine the respective contents of GSH and GSSG in 50 samples.

$$2GSH + DTNB \longrightarrow GSSG + 2TNB$$

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP^+ + 2GSH$$

$$DTNB + H^+ + NADPH \xrightarrow[GSSG/GSH]{GR} 2TNB + NADP^+$$

Components and Storage

Components \ Size	100 T	Storage
Total Glutathione Assay Buffer	70 mL	-20°C
FAD	100 μL	-20°C

DTNB (Ready-to-use)	660 μL	-20°C away from light
GSH Clearing Auxiliary Solution	2 mL	-20°C
GSH Clearing Reagent	500 μL	-20°C
GSSG	5 mg	4°C
Glutathione Reductase (70X)	10 μL	4°C
Protein Removal Reagent M	1 g	4°C
NADPH	4 mg	4°C
Shipping: Blue ice	**Shelf life: 12 months**	

Protocol

1. Self-provided

   96-well plate, microplate reader (405-415 nm, optimal detection wavelength 412 nm), PBS, Milli-Q grade pure water, pipette tips, EP tubes, anhydrous ethanol, vortex mixer, etc.

2. Preparation of the kit:

   a. Preparation of 1X Glutathione Reductase: Take an appropriate amount of Glutathione Reductase (70X) and dilute it to 1X with Total Glutathione Assay Buffer for later use. For example, take 1 μL of Glutathione Reductase (70X) and add 69 μL of Total Glutathione Assay Buffer. After mixing evenly, it becomes 1X Glutathione Reductase.

***Note:** Glutathione Reductase (70X) is stored in the form of suspended particles in saturated ammonium sulfate. Thoroughly mix it before dilution. Use it immediately after dilution and do not let it stand for a long time.

   b. Preparation of GSSG stock solution (10 mM): Add 816 μL of Total Glutathione Assay Buffer to the 5 mg of GSSG provided in this kit, dissolve and mix well. This will yield the GSSG stock solution with a concentration of 10 mM. Except for the portion to be used immediately, aliquot the remaining GSSG stock solution and store at -20°C.

***Note:** It is recommended to aliquot the prepared GSSG stock solution and store it at -20°C. It can be stored for 4-8 weeks.

   c. Preparation of Protein Removal Reagent M solution: Weigh 0.2 g of Protein Removal Reagent M and then add 4 mL of Milli-Q grade pure water to prepare a 4 mL aqueous solution with a concentration of 5%. It is recommended to prepare and use it immediately.

***Note:** Protein Removal Reagent M is a toxic component. The solution preparation should be carried out in a fume hood.

   d. Preparation of DTNB stock solution: DTNB (Ready-to-use) is a ready-to-use reagent. It is recommended to aliquot it for storage to reduce the negative impact of repeated freeze-thaw cycles. It can be directly added to the system during use.

   e. Preparation of NADPH stock solution (40 mg/mL): Add 100 μL of Milli-Q grade pure water to the 4 mg of NADPH provided in this kit, dissolve and mix well to obtain the NADPH stock solution. Take an

appropriate amount of the NADPH stock solution for subsequent experiments, and aliquot the remaining solution and store it at -70°C.

f. Preparation of NADPH working solution (0.5 mg/mL): Take 10 μL of the NADPH stock solution (40 mg/mL) and add 790 μL of Total Glutathione Assay Buffer, mix well to obtain a 0.5 mg/mL NADPH working solution.

***Note:** It is recommended to prepare the NADPH working solution (0.5 mg/mL) when incubating at 25°C for 5 minutes after thorough mixing in the determination of samples and standards (5.a).

g. Preparation of GSH clearing auxiliary solution: Add 53 μL of GSH Clearing Auxiliary Solution to 47 μL of Milli-Q grade pure water and mix immediately.

***Note:** The diluted GSH clearing auxiliary solution is not very stable. It must be freshly prepared each time it is used and is only valid for use on the same day.

h. Preparation of GSH clearing reagent working solution: Add 89.2 μL of anhydrous ethanol to 10.8 μL of GSH Clearing Reagent and mix immediately. The GSH clearing reagent working solution must be freshly prepared each time.

***Note:** GSH Clearing Reagentis a toxic component and should be prepared in a fume hood.

i. Preparation of the Total Glutathione detection working solution: Refer to the following table to prepare an appropriate amount of the Total Glutathione detection working solution according to the number of samples to be tested. The Total Glutathione detection working solution is obtained by mixing the four reagents in the table in proportion.

Components	1 Test	10 Test
Total Glutathione Assay Buffer	140 μL	1.4 mL
DTNB	6.6 μL	66 μL
1X Glutathione Reductase	6.6 μL	66 μL
FAD	1 μL	10 μL

3. Preparation of the standard：

a. Take the GSSG stock solution (10 mM) and dilute it with Protein Removal Reagent M solution to a 50 μM GSSG solution. Then, further dilute it successively to prepare GSSG solutions with gradients of 15, 10, 5, 2, and 1 μM.

***Note:**Since GSSG is not very stable in Protein Removal Reagent M solution, the GSSG solution prepared with Protein Removal Reagent M solution must be used immediately after fresh preparation and cannot be used after being frozen and stored.

b. If the GSSG content in the sample needs to be determined, add the GSH clearing auxiliary solution to the standard solution at a ratio of 20 μL of GSH clearing auxiliary solution per 100 μL of standard solution, and vortex mix immediately. Then, add the GSH clearing reagent working solution to the standard solution at a ratio of 4 μL of GSH clearing reagent working solution per 100 μL of standard solution, and vortex mix immediately. React at 25°C for 60 minutes. The resulting solution can then be used for the subsequent detection of GSSG content.

***Note:** The standard curve directly detected under 3.a is the standard curve for Total Glutathione content. If the operation in 3.b (GSH removal) is added based on 3.a, then the detected standard curve is the standard curve for GSSG content.

4. Sample Preparation

■ Type I test samples: Samples for which the Total Glutathione content needs to be detected

a. Preparation of tissue samples: Take the tissue and rapidly freeze it in liquid nitrogen, and then grind it into powder. Add 30 μL of Protein Removal Reagent M solution to every 10 mg of ground tissue powder and vortex thoroughly. Then add 70 μL of Protein Removal Reagent M solution and homogenize thoroughly with a glass homogenizer (for tissues that are relatively easy to homogenize, the liquid nitrogen freezing and other treatments can be skipped, and an appropriate amount of Protein Removal Reagent M solution can be directly added for homogenization). After standing at 4°C for 10 minutes, centrifuge at 10,000 g and 4°C for 10 minutes, and take the supernatant for the determination of Total Glutathione. The samples need to be temporarily stored at 4°C. Samples that are not measured immediately can be stored at -70°C, but should not exceed 10 days. For the prepared tissue samples, they usually need to be appropriately diluted with Protein Removal Reagent M solution before measurement. The dilution factor is usually 5-20 times.

b. Preparation of cell samples: Wash the cells once with PBS, centrifuge to collect the cells, and aspirate the supernatant completely. Add a volume of Protein Removal Reagent M solution that is 3 times the volume of the cell pellet. That is, if the cell pellet is 10 μL, add 30 μL of Protein Removal Reagent M solution and vortex thoroughly. Then, subject the samples to two rapid freeze-thaw cycles using liquid nitrogen and a 37°C water bath. Place at 4°C or on ice for 5 minutes. Finally, centrifuge at 10,000 g and 4°C for 10 minutes, and take the supernatant for the determination of Total Glutathione. The samples need to be temporarily stored at 4°C. Samples that are not measured immediately can be stored at -70°C, but should not exceed 10 days. For the prepared cell samples, they usually need to be appropriately diluted with Protein Removal Reagent M solution before measurement. The dilution factor can be as high as 20 times.

***Note:** Please try to use fresh cells for determination instead of using frozen cells. The volume of the cell pellet can be estimated based on the weight of the cell pellet. For example, the volume of a 10-milligram cell pellet can be roughly regarded as 10 μL.

c. Preparation of red blood cell or plasma samples. Please try to use fresh blood for determination. Centrifuge at 600 g for 10 minutes. The precipitate is red blood cells, and the supernatant is plasma. For red blood cells, wash them twice with PBS. Take about 50 μL of red blood cell precipitate or plasma, add 50 μL of Protein Removal Reagent M solution, vortex thoroughly, and then place at 4°C or on ice for 10 minutes. Finally, centrifuge at 10,000 g and 4°C for 10 minutes, and take the supernatant for subsequent Total Glutathione determination. The samples need to be temporarily stored at 4°C. Samples that are not measured immediately can be stored at -70°C, but should not exceed 10 days. For the prepared red blood cell samples, they need to be diluted 10 times with Protein Removal Reagent M solution before subsequent determination. For plasma samples, take 10 μL directly for determination.

d. For some samples with particularly low glutathione content, they can be concentrated by freeze-drying and then measured.

■ Type II test samples: Samples for which the GSSG content needs to be detected

Take a portion of the above-prepared samples for Total Glutathione content determination (Type I test samples). Add GSH clearing auxiliary solution to the samples at a ratio of 20 μL of GSH clearing auxiliary solution per 100 μL of sample, and vortex mix immediately. Then, add GSH clearing working solution to the samples at a ratio of 4 μL of GSH clearing working solution per 100 μL of sample, and vortex mix immediately. React at 25°C for 60 minutes. The above reaction can remove up to 50 μM of GSH. If the GSH content in the samples is too high, the samples need to be appropriately diluted before the GSH removal operation. After the above treatment, the samples can be used for subsequent determinations.

5. Determination of samples and standards：

a. Refer to the following table, add samples or standards to a 96-well plate in sequence, mix well, and then add 150 μL of the Total Glutathione detection working solution, mix well. Incubate at 25°C or room temperature for 5 minutes.

	Blank control	Sample	Standard
Protein Removal Agent M solution	10 μL	0 μL	0 μL
Sample	0 μL	10 μL	0 μL
Standard	0 μL	0 μL	10 μL
Glutathione working solution	150 μL	150 μL	150 μL
	After mixing thoroughly, incubate at 25°C for 5 min		
NADPH working solution（0.5 mg/mL）	50 μL	50 μL	50 μL

***Note:**

1. When mixing samples, standards and buffer solution, the mixing time should not be too long, otherwise NADPH will react immediately upon addition.
2. To ensure the accuracy of the experiment, NADPH should be added simultaneously and detected quickly. Generally, a preliminary reaction can occur within 30 s. If the standard curve is tested multiple times, attention should be paid to the reaction time.
3. NADPH and GSSG will show decreased effectiveness with the increase of storage time and number of freeze-thaw cycles. It is normal for the K value of the standard curve to decrease during long-term testing.

b. After the above-mentioned system to be tested is mixed evenly, measure the absorbance immediately with a microplate reader at a wavelength of 412 nm. It is recommended to set a detection interval of 5 minutes or perform real-time measurement, and collect time-series absorbance data at 5 time points within 25 minutes.

***Note:**

1. To simplify the experimental steps, when measuring A_{412} with a microplate reader, only one measurement of A_{412} needs to be taken 25 minutes after adding and mixing the NADPH working solution (0.5 mg/mL).
2. Set the measurement temperature of the microplate reader to 25°C (if it can be set). Otherwise, measure at room temperature. If the microplate reader cannot measure A_{412}, the absorbance in the range of approximately 405-414 nm can be measured.
3. If the standard curve is good but the absorbance of the samples is relatively low, the incubation time can be extended to 30-60 minutes. The absorbance of the standards and samples will increase approximately linearly with time within a certain range.
4. If the GSSG content is to be determined, the standards also need to undergo the relevant operation of removing GSH in parallel to reduce errors. If the Total Glutathione content and GSSG content of the samples need to be determined simultaneously, since the detection systems for the two are different, separate standard curves need to be prepared for each.

6. Calculation of the content of glutathione (Total Glutathione, GSSG or GSH) in the samples:

■ Determination of Total Glutathione or GSSG content

a. Single-point determination method: Measure the absorbance only once after 25 minutes (or 30-60 minutes) of reaction. Prepare a standard curve based on the absorbances measured for standard solutions of different concentrations. The content of Total Glutathione (the GSSG concentration calculated from the standard curve multiplied by 2) or GSSG in the samples can be calculated by comparing with the standard curve. The actual calculated content of Total Glutathione is equivalent to multiplying the content of oxidized glutathione by 2 and then adding the content of reduced glutathione. The single-point determination method is relatively convenient, while the kinetic method is relatively more accurate. Note: Since one molecule of GSSG can be reduced to two molecules of GSH after reaction, when converting the concentration of GSSG to that of GSH, it needs to be multiplied by 2. For example, if the concentration of GSSG is 5 μM after completely removing the endogenous GSH in the samples, it is equivalent to a GSH concentration of 10 μM.

b. Kinetic determination method: First, calculate ΔA_{412}/min based on the absorbance values measured at different time points. Then, plot a standard curve with the concentration of the standards as the x-axis and ΔA_{412}/min as the y-axis. Based on the ΔA_{412}/min of the samples and by referring to the standard curve, the content of Total Glutathione or GSSG in the samples at the time of determination can be calculated.

c. At the same time, calculate the content of Total Glutathione per milligram of tissue or cells based on the dilution factor of the samples and the initial amount of the samples used. For cell samples, the protein content in the cell samples can also be calculated as follows: Based on the initial number of cells used, take a certain number of cells and lyse them, then measure the protein concentration to obtain the protein amount in the cell samples. Finally, calculate the content of Total Glutathione or GSSG per milligram of protein.

■ Determination of GSH content

Method: The content of GSH can be calculated based on the measured contents of Total Glutathione and GSSG.

Reference calculation formula: GSH = Total Glutathione - GSSG × 2 (Note: The Total Glutathione is obtained by multiplying the GSSG concentration calculated from the standard curve by 2. Also, the GSSG obtained after removing GSH should be multiplied by 2, because one molecule of GSSG can be reduced to two molecules of GSH after reaction). For example, the concentration of Total Glutathione measured by this kit is 15 μM (that is, the GSSG concentration calculated from the standard curve during the determination of Total Glutathione is 7.5 μM, and multiplying it by 2 gives the Total Glutathione concentration). The measured concentration of GSSG is 1.2 μM (that is, the GSSG concentration calculated from the standard curve during the separate determination of GSSG content is 1.2 μM). Then the concentration of GSH in the sample is 15 - 1.2 × 2 = 12.6 μM.

Note

1. This kit can be used to detect the content of total glutathione in animal tissues, plasma, red blood cells, cultured cells, or other appropriate samples.
2. The Protein Removal Reagent M solution must be freshly prepared and used on the same day. If a larger quantity is needed, it can be purchased separately. The product code is H1004. This product is not very soluble. It is recommended to promote dissolution by vigorous vortexing and appropriate heating (not exceeding 37°C).
3. It is recommended to use the GSH Clearing Auxiliary Solution as soon as possible after dilution.
4. FAD is a coenzyme for reductases and should be added to the reaction system along with the reductase during use.
5. Redox reactions are involved in the detection process of this kit. Therefore, all oxidizing agents or reducing agents will interfere with the determination of this kit. In particular, reagents containing thiol groups such as DTT and mercaptoethanol will seriously interfere with the determination of this kit. Please try to avoid using them.
6. The temperature and reaction time during the reaction must be strictly controlled. Otherwise, the standard curve needs to be prepared each time.
7. Reagents such as NADPH are not very stable. Please operate strictly according to the subsequent instructions to prevent inactivation.
8. This product is for scientific research use only.

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