Protocol

Cat. No. K4629

Total Glutathione Detection Kit (DTNB Colorimetric Method)

Introduction

Glutathione is a small peptide composed of 3 amino acid residues, the full name of which is glutamyl-cysteinyl-glycine. Because the sulfhydryl group (SH) on cysteine is the active group of glutathione, it is often abbreviated as G-SH or GSH. There are two forms of glutathione: oxidized glutathione disulfide and reduced glutathione (commonly known as GSH). Oxidized glutathione is produced by the dehydrogenation of two GSHs by sulfhydryl groups, often abbreviated as G-S-S-G or GSSG. Reduced glutathione is the main source of sulfhydryl groups in the vast majority of living cells, plays an important role in maintaining the proper redox state of sulfhydryl groups in proteins, and is a key antioxidant in animal cells. Typically, 90-95% of total glutathione is reduced glutathione.

In the detection of total glutathione, first glutathione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH), and then GSH reacts with DTNB to produce yellow TNB, and the amount of total glutathione determines the amount of yellow TNB formation, and the amount of total glutathione can be obtained by detecting the absorbance of A_{412}.

This kit is a simple and easy to measure total glutathione (GSSG+GSH) for the determination of total glutathione in animal tissues, plasma, red blood cells, cultured cells, or other appropriate 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℃
FAD	100 μL	-20℃
DTNB (Ready-to-use)	660 μL	-20°C away from light
Reduced Glutathione (GSH)	4.5 mg	-20°C away from light
GSH Buffer	1.5 mL	4℃
Glutathione Reductase (70X)	10 μL	4℃
Protein Removal Reagent S	0.4 g	4℃
NADPH	4 mg	4°C away from light

Shipping: Blue ice	Shelf life: 12 months

Protocol

1. Materials not-Supplied：

   96-well plates, microplate readers (405-415 nm, optimal detection wavelength 412 nm), PBS, Milli-Q pure water, pipettes, EP tubes, vortex mixers, etc

2. Kit preparation:

   a. Preparation of GSH stock solution (10 mM): Add 1.5 mL of GSH Buffer to the 4.5 mg Reduced Glutathione (GSH) provided in this kit and mix well to form a GSH stock solution at a concentration of 10 mM. Take an appropriate amount of GSH stock solution for subsequent experiments, and store the rest at 4°C after aliquoting.

***Note:** GSH Buffer is a specially formulated solution provided by our company. Reduced Glutathione (GSH) can be stored at 4℃ for 3-6 months after dilution with this buffer.

   b. Preparation of Protein Removal Reagent S Solution (5%): Add 8 mL of Milli-Q water to 0.4 g of Protein Removal Reagent S provided in the kit to prepare an 8 mL 5% aqueous solution. Store at 4℃.

***Note:** Protein Removal Reagent S is a toxic component and should be prepared in a fume hood.

   c. DTNB: DTNB (Ready-to-use) is a ready-to-use solution that can be added directly to the detection system. It is recommended to store at -20°C in aliquots.

   d. Preparation of NADPH stock solution (40 mg/mL): NADPH stock solution is dissolved and mixed in 100 µL of Milli-Q grade pure water in the 4 mg NADPH provided in this kit. Take an appropriate amount of NADPH stock solution for subsequent experiments, and store the rest at -70°C after aliquoting. 50 µL of 0.5 mg/mL NADPH per sample tested.

   e. Preparation of NADPH Working Solution (0.5 mg/mL): Take 10 µL of 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:** The NADPH working solution (0.5 mg/mL) is recommended to be configured after thorough mixing in the assay of samples and standards (5.a) and incubated at 25°C for 5 minutes.

   f. Preparation of 1X Glutathione Reductase: Take 1 µL of Glutathione Reductase (70X) and add 69 µL of Total Glutathione Assay Buffer, mix well to obtain 1X reductase.

***Note:** Glutathione Reductase (70X) is stored as a suspension in saturated ammonium sulfate. It must be thoroughly mixed before dilution. The diluted solution should be used immediately and not stored for long periods.

   g. Preparation of total glutathione detection working solution: according to the number of samples to be tested, refer to the following table to prepare an appropriate amount of total glutathione detection working solution, and the four reagents in the table are mixed in proportion to be the total glutathione

detection working solution.

Component	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 GSH standard: Appropriate amount of GSH stock solution (10 mM) was diluted to 50 μM with protein removal reagent S solution (5%), and then diluted to 25, 15, 10, 5, and 2 μM. Six points of 50, 25, 15, 10, 5, and 2 μM GSH solution were taken as standard curves.

***Note:** Because GSH stock solutions are less stable in protein depletion Reagent S solution, GSH solutions prepared with protein depletion Reagent S solution must be freshly prepared for use and not cryopreserved for use.

4. Preparation of samples:

   a. Preparation of tissue samples: The tissue is quick-frozen with liquid nitrogen and then ground into powder. Add 30 μL of Protein Depletion Reagent S (5%) solution per 10 mg of ground tissue powder and fully Vortex. Then add 70 μL of protein removal reagent S solution (5%), and use a glass homogenizer to homogenize fully (for tissues that are easy to homogenize, you can directly add an appropriate amount of protein removal reagent S solution (5%) for homogenization without liquid nitrogen quick freezing, etc.). After 10 minutes at 4°C, centrifuge at 10,000 g at 4°C for 10 minutes, and the supernatant was taken for the determination of total glutathione. Samples should be stored at 4°C temporarily, and samples that are not measured immediately can be stored at -70°C, but not for more than 10 days. Processed tissue samples are usually appropriately diluted with protein removal reagent S solution (5%) before being measured, typically in 5-20-fold dilutions.

   b. Preparation of cell samples: take the cells and wash them once with PBS, centrifuge to collect the cells, and aspirate the supernatant. Add 3 times the volume of the cell pellet protein depletion Reagent S solution (5%), i.e., if the cell pellet is 10 μL, add 30 μL of protein depletion Reagent S solution (5%), fully vortex. The samples were then subjected to two rapid freeze-thaw attempts using liquid nitrogen and a 37°C water bath, 5 minutes at 4°C or in an ice bath, and finally 10,000 g centrifugation at 4°C for 10 minutes, and the supernatant was used for the determination of total glutathione. Samples should be stored at 4°C temporarily, and samples that are not measured immediately can be stored at -70°C, but not for more than 10 days. Processed cell samples are typically appropriately diluted with protein removal reagent S (5%) before being assayed, and dilutions can be up to 20-fold.

***Note:** Try to use fresh cells instead of cryopreserved; The volume of the cell pellet can be estimated based on the weight of the cell pellet, e.g., the volume of 10 mg of cell pellet can be roughly considered as 10 μL.

   c. Preparation of red blood cell or plasma samples. Please use fresh blood for the test as much as possible. Centrifugation at 600g for 10 min, the pellet was red blood cells, and the supernatant was plasma. For red blood cells, wash twice with PBS. Approximately 50 μL of erythrocyte pellet or plasma

was taken and 50 μL of protein depletion Reagent S solution (5%) was added, and Vortex was fully equipped. Place at 4°C or in an ice bath for 10 minutes. Centrifuge at 10,000g for 10 min at 4°C. The supernatant is taken for the determination of total glutathione. Samples should be stored at 4°C temporarily, and samples that are not measured immediately can be stored at -70°C, but not for more than 10 days. Processed red blood cell samples should be diluted 10-fold with protein depletion Reagent S (5%) before subsequent assays, while plasma samples should be measured directly by 10 μL.

d. Some samples with particularly low glutathione content can be concentrated by freeze-drying before being measured.

5. Determination of samples and standards:

a. Refer to the table below, use a 96-well plate to add samples or standards sequentially and mix well, then add 150 μL of total glutathione detection solution to mix, and incubate at 25°C or room temperature for 5 minutes.

	Blank control	Sample	Standard
Protein Removal Reagent S Solution (5%)	10 μL	0 μL	0 μL
Sample	0 μL	10 μL	0 μL
GSH Standard	0 μL	0 μL	10 μL
Total Glutathione Detection 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. It is recommended to prepare NADPH working solution (0.5 mg/mL) (2.e) after thorough mixing in 5.a, incubate at 25°C for 5 min, and then add subsequently.
2. The sample, GSH standard and protein removal reagent S solution (5%) should be mixed for too long, otherwise the addition of NADPH will immediately cause the reaction.
3. In order to ensure the accuracy of the experiment, NADPH working solution (0.5 mg/mL) should be added at the same time for rapid detection, and the initial reaction can be achieved in 30 s.
4. NADPH will decrease with storage time, freeze-thaw times, and it is normal for the K value of the calibration to decrease for a long time.

b. After thorough mixing, A_{412} was measured immediately with a microplate reader, every 5 min or in real time, for a total of 25 min, and 5 data were measured.

***Note:**

1. In order to simplify the experimental procedure, A_{412} can be measured only once by adding NADPH working solution (0.5

mg/mL) and mixing 25 minutes after the microplate reader determines A_{412}.

2. The microplate reader assay temperature is set at 25°C (if it can be set), otherwise it is determined at room temperature. If the microplate reader is not able to measure A_{412}, the absorbance in the range around 405-414 nm can be determined.
3. If the standard curve is good, but the absorbance of the sample is low, the incubation time can be extended to 30-60 minutes, and the absorbance of the standard and sample will increase nearly linearly over time over a certain range.
4. If the sample absorbance plateaus within 25 min during the first measurement, dilute the sample further with Protein Removal Reagent S and retest.

6. Calculation of total glutathione content in a sample:

   a. Single-point assay: Absorbance is measured only once after 25 minutes (or 30-60 minutes) of the reaction. Standard curves were made based on the different absorbances measured by different concentration standards. The total glutathione content can be calculated by comparing the sample to the standard curve. The actual calculated total glutathione content is equivalent to multiplying the amount of oxidized glutathione by 2 plus the amount of reduced glutathione. The single-point method is relatively convenient, while the kinetic method is relatively accurate.

   b. Kinetic assay: ΔA_{412}/min is calculated based on the absorbance values measured at different time points. Then, the concentration of the standard was used as the abscissa and ΔA_{412}/min was used as the ordinate to make the standard curve. Based on the ΔA_{412}/min of the sample, the total glutathione content in the sample at the time of determination can be calculated against the standard curve.

   c. The amount of total glutathione per milligram of tissue or cell was calculated based on the dilution factor of the sample and the amount of initial sample used. For cell samples, the protein concentration can also be calculated based on the initial number of cells used, and then an additional number of cells lysed to calculate the amount of protein in the cell sample, and finally the total glutathione content per milligram of protein.

Note

1. This kit measures the total glutathione in animal tissues, plasma, red blood cells, and cultured cells, or other appropriate samples.
2. This kit provides protein depletion reagent S to more accurately determine the amount of total glutathione in a sample containing protein.
3. The lower limit of detection for this kit is 1 μM. A total of 100 tests can be performed with one kit.
4. The detection of this kit involves redox reactions, so all oxidants or reducing agents will interfere with the determination of this kit, especially DTT, mercaptoethanol and other reagents containing sulfhydryl groups will seriously interfere with the determination of this kit, please try to avoid it.
5. The temperature and reaction time of the reaction must be strictly controlled, otherwise a standard curve

needs to be made every time.

6. NADPH and other reagents are not very stable, so it is necessary to operate strictly according to the follow-up instructions to prevent inactivation.

7. This product is for scientific research use only.

APExBIO Technology
www.apexbt.com
7505 Fannin street, Suite 410, Houston, TX 77054.
Tel: +1-832-696-8203 | Fax: +1-832-641-3177 | Email: info@apexbt.com