

# Total Glutathione Quantification Kit

## Technical Manual

Product Code: T419-10

### Contents

General Information .....	1
Contents of the Kit .....	1
Storage .....	1
How to Use Total Glutathione Quantification Kit .....	1
1. Required Equipments and Materials .....	1
2. Protocol .....	2
Interference.....	4
Notes .....	4
Product Code and Price .....	4
Related Products .....	4
References .....	4
General Protocol at a Glance .....	5

## GENERAL INFORMATION

Glutathione (GSH) is the most abundant thiol (SH) compound in animal tissues, plant tissues, bacteria and yeast. GSH plays many different roles such as protection against reactive oxygen species and maintenance of protein SH groups. During these reactions, GSH is converted into glutathione disulfide (GSSG: oxidized form of GSH). Since GSSG is enzymatically reduced by glutathione reductase, GSH is the dominant form in organisms.

DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)), known as Ellman's Reagent, was developed for the detection of thiol compounds. In 1985, Dr. Anderson suggested that the glutathione recycling system by DTNB and glutathione reductase created a highly sensitive glutathione detection method. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the measurement at 412 nm absorbance. GSH is generated from GSSG by glutathione reductase, and reacts with DTNB again to produce 2-nitro-5-thiobenzoic acid. Therefore, this recycling reaction improves the sensitivity of total glutathione detection (Fig. 1).

**Total Glutathione Quantification Kit** includes all the necessary reagents for total glutathione measurement except for reagents that are used for sample preparation. 5-Sulfosalicylic acid is recommended for the removal of proteins from sample solutions and for the prevention of GSH oxidation and  $\gamma$ -glutamyl transpeptidase reaction. However, the optimum method for sample preparation differs from sample to sample, so please review the references. This kit can be used to quantify total glutathione concentrations from 1  $\mu$ M to 100  $\mu$ M using the standard method. For lower glutathione concentration detections, such as in blood samples, longer incubation time is required.

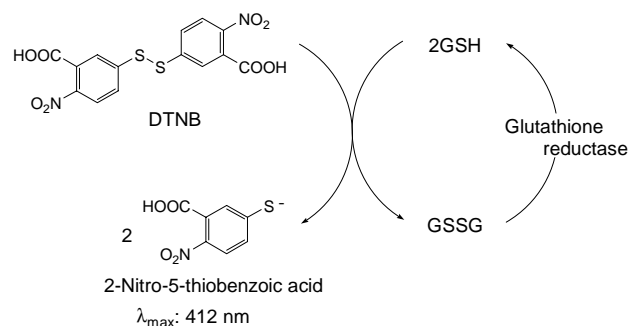


Figure 1. Principle of Total Glutathione Quantification Kit

Figure 2 shows the absorption spectrum of 2-nitro-5-thiobenzoic acid in a phosphate buffer at pH 7.5. Since the maximum absorption is at 412 nm, please use either 405 nm filter or 415 nm filter for the glutathione assay.

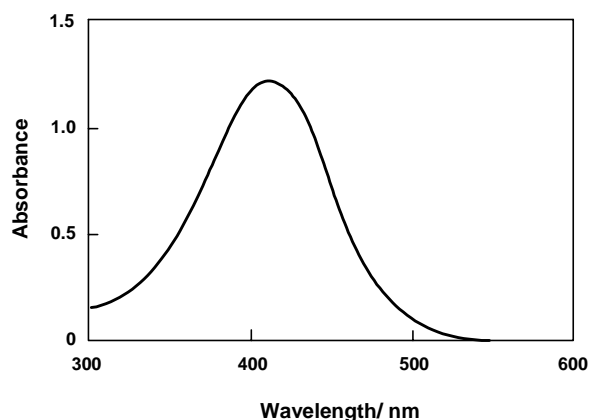


Figure 2. Absorption spectrum of 2-nitro-5-thiobenzoic acid

## KIT CONTENTS

- Substrate (DTNB)	2 vials
- Enzyme Solution	50 $\mu$ l x 1 vial
- Co-enzyme (lyophilized form)	2 vials
- Standard GSH (lyophilized form)	1 vial*
- Buffer solution	50 ml x 1
- Manual	1 booklet

\* The Standard GSH vial contains 0.134 mg of lyophilized GSH, thus, it may be difficult to see the powder.

## STORAGE

Store the Kit at 0-5  $^{\circ}$ C. It is stable for 1 year at 0-5  $^{\circ}$ C. Both Substrate Working Solution and GSH Standard Solution can be stored at -20  $^{\circ}$ C for 2 months. Enzyme Working Solution is stable for 2 months at 4  $^{\circ}$ C (Do not freeze). Coenzyme Working Solution is not stable. Use up the entire solution within one day.

## HOW TO USE THE KIT

### 1. Required Equipments and Materials

- Plate reader (with 405nm filter or 415nm filter)
- 96-well microplate
- 20  $\mu$ l, 200  $\mu$ l and multi-channel pipettes
- Incubator (30  $^{\circ}$ C)
- 5-Sulfosalicylic acid (SSA)

## 2. Protocol

### Preparation of Sample Solution

Following methods are typical sample preparation procedures for the total glutathione assay. Modifications may be necessary. For more info on detailed conditions, see the reference #3 (Anderson, 1985).

#### 1. Preparation of 5% 5-sulfosalicylic acid (5% SSA; not included in this kit):

Mix 1 g of SSA with 19 ml of water.

Store the solution at 4 °C (stable for 6 months at 4 °C).

#### 2. Preparation of Sample Solution:

##### • Cell (adhesive cell: $5 \times 10^5$ cells, leukocyte cell: $1 \times 10^6$ cells)

- 1) Collect cells by centrifugation at 200 x g for 10 min at 4 °C, and discard the supernatant.
- 2) Wash the cells with 300  $\mu$ l PBS, and centrifuge at 200 x g for 10 min. at 4 °C. Discard the supernatant.
- 3) Add 80  $\mu$ l of 10 mM HCl, and lyse the cells by freezing and thawing (x 2).
- 4) Add 20  $\mu$ l of 5 % SSA, and centrifuge at 8000 x g for 10 min.
- 5) Transfer the supernatant to a new tube, and use it for the total glutathione assay <sup>a)</sup>.

##### • Tissue (weight: 100 mg)

- 1) Homogenize the tissue in 0.5 - 1 ml of 5 % SSA.
- 2) Centrifuge the homogenized tissue sample at 8000 x g for 10 min.
- 3) Transfer the supernatant to a new tube, and use it for the total glutathione assay <sup>a)</sup>.

##### • Plasma

- 1) Centrifuge an anticoagulant treated blood at 1000 x g for 10 min at 4 °C.
- 2) Transfer the top plasma layer to a new tube and add 1/2 vol of 5 % SSA.
- 3) Centrifuge at 8000 x g for 10 min at 4 °C
- 4) Transfer the supernatant to a new tube, and use it for the total glutathione assay <sup>a)</sup>.

##### • Erythrocyte <sup>b)</sup>

- 1) Centrifuge an anticoagulant treated blood at 1000 x g for 10 min at 4 °C.
- 2) Discard the supernatant and the white buffy layer.
- 3) Lyse the erythrocytes with 4 vol of 5 % SSA.
- 4) Centrifuge at 8000 x g for 10 min at 4 °C.
- 5) Transfer the supernatant to a new tube, and use it for the glutathione assay <sup>a)</sup>.

<sup>a)</sup> Prior to using the sample solution, please dilute it with water to adjust the concentration of SSA at 0.5 - 1 %. High concentrations of SSA cause pH changes and interfere with the glutathione assay reaction.

<sup>b)</sup> Erythrocytes can be isolated from the remaining sample solution after the plasma sample isolation.

### Preparation of Solutions

#### • Substrate working solution

Add 1 ml of Buffer Solution to one vial of Substrate, and dissolve.

Store the remaining solution at -20 °C (stable for 2 months at -20 °C).

#### • Enzyme working solution

Mix Enzyme Solution using pipette. Take out 20  $\mu$ l of Enzyme solution, and mix it with 4 ml of Buffer solution.

Store the remaining solution at 4 °C (stable for 2 months at 4 °C).

#### • Coenzyme working solution

Add 7.0 ml Buffer solution to the Coenzyme vial, and dissolve.

The Coenzyme vial is decompressed; carefully open the cap or use a syringe to add the Buffer solution.

Use up the solution within one day.

#### • GSH standard solutions

Add 2 ml of 0.5 - 1 % SSA to the Standard GSH vial, and dissolve to prepare 200  $\mu$ M GSH standard solution.

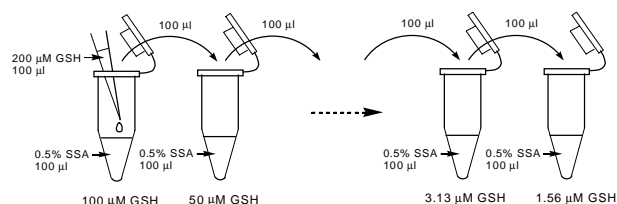
The Standard GSH vial is decompressed; carefully open the cap or use a syringe to add SSA.

Store the remaining solution at -20 °C (stable for 2 months at -20 °C).

Dilute 100  $\mu$ l of 200  $\mu$ M GSH standard solution by serial dilution with 100  $\mu$ l of 0.5% SSA in plastic tubes as indicated in the following figure.

Use up the solution within one day.

GSH standard solutions: 100  $\mu$ M, 50  $\mu$ M, 25  $\mu$ M, 12.5  $\mu$ M, 6.25  $\mu$ M, 3.13  $\mu$ M, 1.56  $\mu$ M and 0  $\mu$ M.



### General Protocol (illustrated on page 5)

#### Total glutathione detection (standard method).

Detection range: 5 - 100  $\mu$ M

- 1) To each well, add 20  $\mu$ l of Enzyme working solution, 140  $\mu$ l of Coenzyme working solution and 20  $\mu$ l of either one of the GSH standard solutions or the sample solution.<sup>a)</sup>

<sup>a)</sup> Adjust the concentration of SSA in the sample solution to 0.5 - 1 % with ddH<sub>2</sub>O before the assay. High concentrations of SSA (>1 %) interfere with the assay. See next page for a plate diagram of the solution arrangement.

- 2) Incubate the plate at 37 °C for 10 min.
- 3) Add 20  $\mu$ l of Substrate working solution, and incubate the plate at room temperature for 5-10 min.

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank											
B	1.56 $\mu$ M GSH											
C	3.13 $\mu$ M GSH											
D	6.25 $\mu$ M GSH			Sample								
E	12.5 $\mu$ M GSH											
F	25 $\mu$ M GSH											
G	50 $\mu$ M GSH											
H	100 $\mu$ M GSH											

Plate Diagram: an example of the GSH standard solution and sample solution arrangement to be measured in triplicate (up to 24 samples can be determined in one plate).

- 4) Read the absorbance at 405 nm or 415 nm using a microplate reader.
- 5) Determine concentrations of GSH in the sample solutions using a calibration curve<sup>b</sup>.

*b) Since the colorimetric reaction is stable and the O.D. increases linearly over 30 min, GSH concentration can be determined by using kinetic or pseudo-endpoint (no stopping reaction, quick measurement of the O.D. at certain time points between 5 and 10 min) methods. A time course of the colorimetric reaction is shown in figure 4. Typical calibration curves prepared using the kinetic method and the pseudo-endpoint method are indicated in Figure 5 and 6, respectively.*

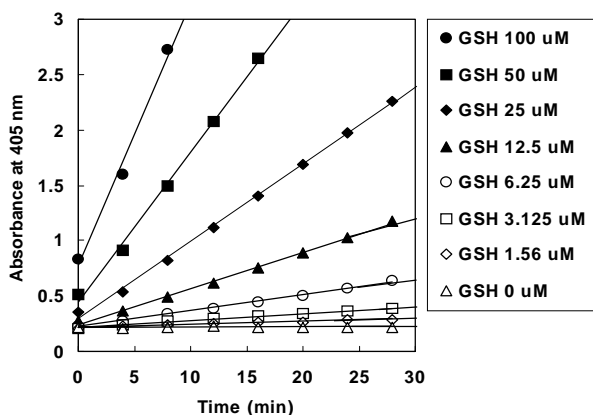


Figure 4. Time course of DTNB colorimetric reaction\* in various concentrations of GSH.

\* Reaction time was measured immediately after the addition of Substrate working solution.

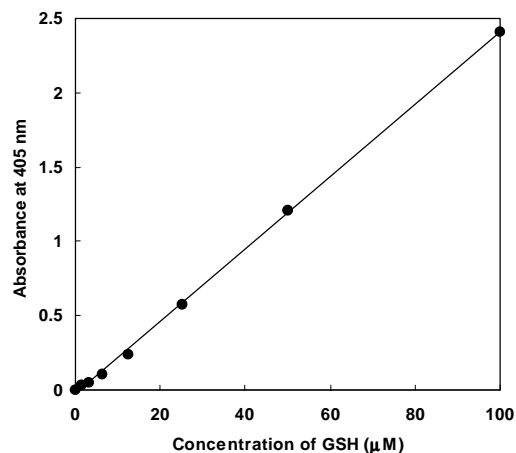


Figure 5. Calibration curve prepared using pseudo-end point method (10 min incubation at room temperature)

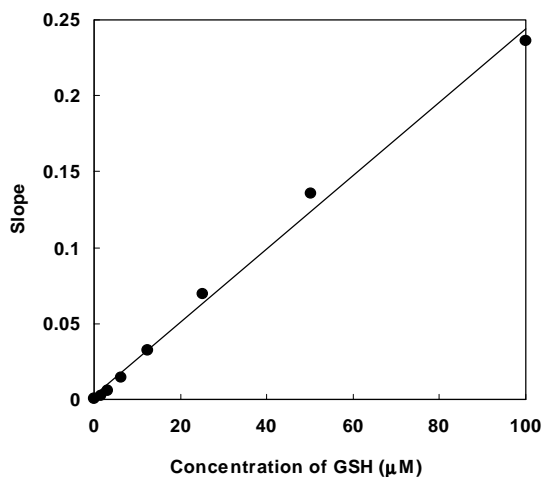


Figure 6. Calibration curve prepared using kinetic method.

Total glutathione detection (high sensitivity method)  
Detection range: 0.5-25  $\mu$ M

- 1) To each well, add 20  $\mu$ l of Enzyme working solution, 140  $\mu$ l of Coenzyme working solution and 20  $\mu$ l of either of one of the GSH standard solutions<sup>a</sup>) or the sample solution<sup>b</sup>).

*a) Prepare 50  $\mu$ M GSH standard solution, and then prepare different concentrations of GSH standard solutions by serial dilution with 0.5% SSA as follows: 25  $\mu$ M, 12.5  $\mu$ M, 6.25  $\mu$ M, 3.13  $\mu$ M, 1.56  $\mu$ M, 0.78  $\mu$ M, 0.39  $\mu$ M and 0  $\mu$ M.*

*b) Adjust the concentration of SSA in the sample solution to 0.5 - 1 % with ddH<sub>2</sub>O before the assay. High concentrations of SSA (>1 %) interfere with the assay. A plate diagram of the solution arrangement is shown on the left-side column of this page.*

- 2) Incubate the plate at 30  $^{\circ}$ C for 10 min.
- 3) Add 20  $\mu$ l of Substrate working solution, and incubate the plate at 37  $^{\circ}$ C for 20-40 min.

- 4) Read the absorbance at 405 nm or 415 nm using a microplate reader.
- 5) Determine concentrations of GSH in sample solutions using a calibration curve.

#### Calculation of total glutathione (GSH and GSSG) concentration

Determine the total glutathione concentration<sup>a)</sup> in a sample solution using the following equations.

##### Pseudo-end point method:

$$\text{Total glutathione} = (\text{O.D.}_{\text{sample}} - \text{O.D.}_{\text{blank}})$$

##### Kinetic method:

$$\text{Total glutathione} = (\text{Slope}^{\text{c)}}_{\text{sample}} - \text{Slope}^{\text{c)}}_{\text{blank}}) / \text{slope}^{\text{b)}})$$

a) Since the values obtained by these equations are the amount of total glutathione in treated sample solutions, further calculations are necessary if the actual concentrations of glutathione in cells or tissues need to be determined.

b) slope of the calibration curve.

c) slope of the kinetic reaction.

### 3. 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, SH compounds, reducing agents and SH reactive materials should be avoided during the sample preparation.

### NOTES

1. Store the kit at 0 - 5 °C. It is stable for 1 year at 0 - 5 °C.
2. Use the reagents in the kit after the reagents temperatures are equilibrated to the room temperature.
3. Triplicate measurements per sample is recommended to obtain accurate data.
4. Since the colorimetric reaction starts immediately after the addition of Substrate working solution to a well, use a multichannel pipette to avoid the reaction time lag of each well.
5. If the concentration range of total glutathione in a sample is not known, prepare multiple-diluted sample solutions.
6. This kit is not for GSSG determination. For GSSG quantification, please use GSSG instead of GSH standard solution, and quench GSH by using 2-vinylpyridine as indicated in reference # 2 and 3. Add Coenzyme working solution, Enzyme working solution and Substrate solution simultaneously to

the 2-vinylpyridine-treated sample, and incubate the mixture at room temperature for 15 min. Then, read the O.D. at 405 nm or 415 nm. Usually, the GSSG concentrations of biological sample solutions are very low, so even the high sensitivity method may not be enough to quantify the amount of GSSG.

### PRODUCT CODE AND PRICE

Product Code	Product Name	Unit	Price (\$)
T419-10	Total Glutathione Quantification Kit	100 tests	\$175.00

### RELATED PRODUCTS

Product Code	Product Name	Unit	Price (\$)
D029-10	DTNB	1 g	\$34.00
S311-10	SOD Assay Kit-WST	500 tests	\$210.00

### REFERENCES

1. G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
2. O. W. Griffith, *Anal. Biochem.*, **106**, 207 (1980).
3. M. E. Anderson, *Methods in Enzymol.*, **113**, 548 (1985).
4. M. A. Baker, G. J. Cerniglia, and A. Zaman, *Anal. Biochem.*, **190**, 360 (1990).
5. C. Vandeputte, I. Guizon, I. Genestie-Denis, B. Vannier and G. Lorenzon, *Cell Biol. Toxicol.*, **10**, 415 (1994).

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# General Protocol at a Glance

Read Technical Information carefully prior to using this General Protocol

## Step 1-2)



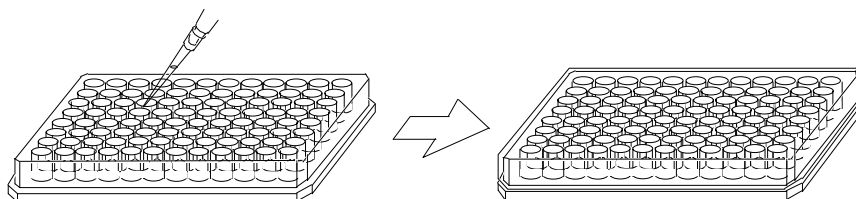
10 min



37 °C

To each well, add 20  $\mu$ l of Enzyme working solution, 140  $\mu$ l of Coenzyme working solution and 20  $\mu$ l of either one of the GSH standard solutions or the sample solution<sup>a)</sup>, and incubate the plate at 37 °C for 10 min.

a) Adjust the concentration of SSA in the sample solution to 0.5 - 1 % with ddH<sub>2</sub>O before the assay. High concentrations of SSA (>1 %) interfere with the assay.

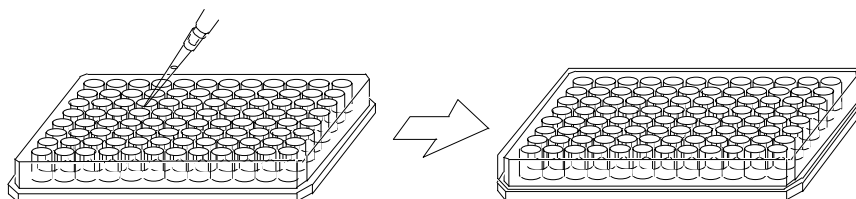


## Step 3)

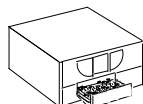


5-10 min

Add 20  $\mu$ l of Substrate working solution, and incubate the plate at room temperature for 5-10 min.



## Step 4-5)



Read the absorbance at 405 nm or 415 nm using a microplate reader, and determine concentrations of GSH in the sample solutions using a calibration curve<sup>b)</sup>.

b) Since the colorimetric reaction is stable and the O.D. increases linearly over 30 min, GSH concentration can be determined by using kinetic or pseudo-endpoint (no stopping reaction, quick measurement of the O.D. at certain time points between 5 and 10 min) methods. A time course of the colorimetric reaction is shown in figure 4. Typical calibration curves prepared using the kinetic method and the pseudo-endpoint method are indicated in Figure 5 and 6, respectively.