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BIOXYTECH®

Glutathione Microplate (GSH-MP™) Assay Kit

Catalog Number 21037
For Research Use Only.
Store at 2 to 8°C.

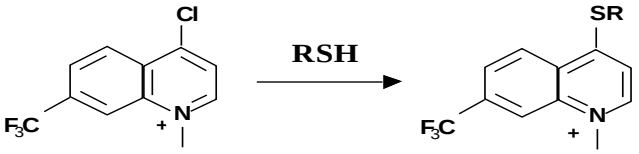
INTRODUCTION

The Analyte

Glutathione (gamma-glutamylcysteinylglycine or GSH) is a naturally occuring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells.[1] Glutathione (GSH) is the most abundant antioxidant in aerobic cells, present in micromolar (microM)-concentrations in bodily fluids and in millimolar (mM) concentrations in tissue. GSH is critical for protecting the brain from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation.[2] GSH plays a critical role as a coenzyme with a variety of enzymes including, glutathione peroxidase, glutathione S-transferase, and thiol transferase. GSH also plays major roles in drug metabolism, calcium metabolism, the γ-glutamyl cycle, blood platelet, and membrane functions. In addition, GSH is crucial to a variety of life processes, including the detoxification of xenobiotics, maintenance of the -SH level of proteins, thiol-disulfide exchange, removal of hydroperoxides and free radicals, and amino acid transport across membranes. Physiological values of intracellular GSH generally range from 1 to 10 mM. Although many methods have been described for the assay of GSH, the reliable ones are labor intensive and not easy to use.[3]

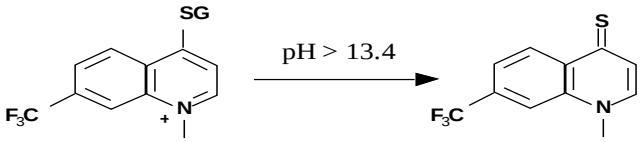
Principles of the Procedure

The GSH-400 method is based on a chemical reaction which proceeds in two steps.[4] The first step leads to the formation of substitution products (thioethers) between a patented reagent, R1 (4-chloro-1-methyl-7-trifluromethyl-quinolinium methylsulfate), and all mercaptans (RSH) which are present in the sample:



Reagent R1 **Thioethers**

The second step is a β-elimination reaction which takes place under alkaline conditions. This reaction is mediated by reagent R2 (30% NaOH) which specifically transforms the substitution product (thioether) obtained with GSH into a chromophoric thione which has a maximal absorbance wavelength at 400 nm:



GSH-thioether **Thione**

The GSH-400 method makes it possible to specifically assay glutathione with only one sampling and one colorimetric measurement. A modification of this method can be used to assay other mercaptans. This is based on the measurement of substitution products, thioethers, which absorb light at 356 nm in the absence of reagent R2.

Because of its simplicity, the GSH-400 method is especially well adapted to the assay of glutathione in large series of biological samples. The main advantage of the method is the specificity for glutathione and it does not require an enzyme as a reagent.

REAGENTS

Materials Provided (for 96 tests)

These three solutions are ready for use.

- Reagent (R1) — Solution of chromogenic reagent in HCl. 1 x 1.1 mL
- 30 % NaOH (R2) — 1 x 4 mL
- Buffer (Solution 3) — Potassium phosphate, containing diethylenetriamine pentaacetic acid (DTPA) and lubrol. 1 x 20 mL
- Microplate — 96-well Microplate, 1 X plate

Materials Required But Not Provided

- Spectrophotometer plate reader with a 400 nm filter (filters between 397 nm or 405 nm are also acceptable).
- Adjustable pipettes with disposable tips.
- Disposable glass test tubes and shake mixer.
- Water bath kept within 22-28°C temperature range.
- Reduced glutathione (GSH), purity >98%.
- Metaphosphoric acid (MPA), purity 33-37%.

Warnings and Precautions

- For in vitro use only.
- Do not smoke, eat, or drink in areas where reagents and samples are manipulated.
- Wear disposable gloves when handling reagents and samples.
- Mouth pipetting is not recommended.
- Avoid skin and eye contact with the reagents R1 and R2.
- Reagent R2 contains 30% sodium hydroxide, which can cause severe burns.
- In case of accidental exposure of skin, eyes, or mucous membranes thoroughly wash the exposed area with water for 15 minutes.

Reagent Storage and Handling

Reagent bottles should always be kept tightly closed and stored at 0-4°C. For each experiment, take out the required amount of buffer and reagents. Transfer should be made by using clean pipette tips to avoid contamination. Immediately close the remaining buffer and reagent bottles, and store them at 0-4°C. Do not leave reagent bottles open on the bench, at room temperature, or exposed to light. Under the above conditions, all reagents are stable until the indicated expiration date.

PROCEDURE

Assay for Standard

Before each new series of assays, prepare a standard curve with at least five distinct concentrations of GSH. These five concentrations should cover the range 20-100 µmol/L in the reaction medium (microplate). Prepare a MPA working solution by dissolving 5 grams MPA in 100

mL water. Prepare a 0.5 mmol/L GSH working standard solution by dissolving GSH into MPA working solution.

Table 1: GSH Standard Curve

[GSH] µmol/L	0 (Blank)	10	**20**	**40**	**60**	**80**	**100**
Buffer S3 (µL)	**180**	**196**	**172**	**164**	**156**	**148**	**140**
GSH 0.5 mmol/L (µL)	**0**	**4**	**8**	**16**	**24**	**32**	**40**

⇓

Add 10 µL of R1

⇓

Shake

⇓

Add 10 µL of R2

⇓

Shake

⇓

Incubate at 25 ± 3°C for 10 minutes in the dark

⇓

Read absorbance at 400 nm

A least-squares linear regression should demonstrate that the absorbance at 400 nm (A) is a linear function of GSH concentration. The apparent molar extinction coefficient, ε, of the measured product is equal to the slope of the corresponding straight line. An example of a standard curve obtained at 400 nm is shown in Figure 1.

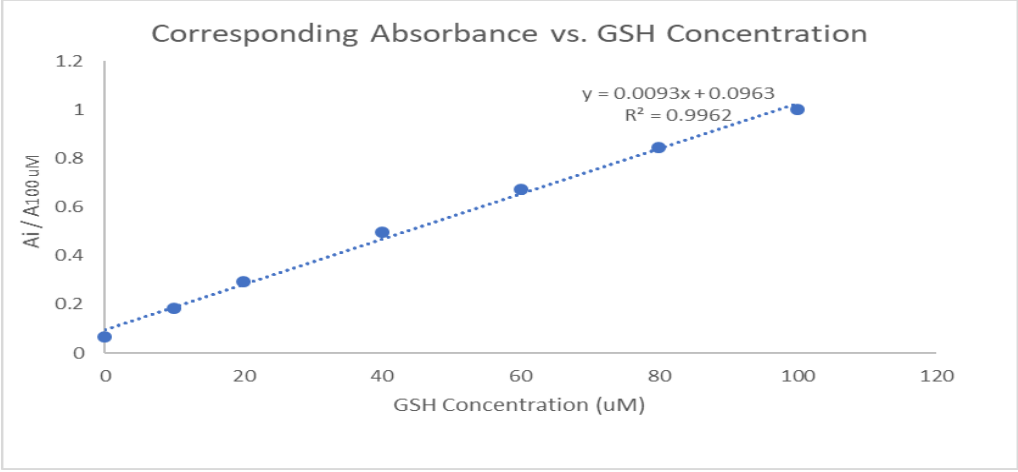


Figure 1: Example of standard curve obtained at 400 nm at 25°C.

Assay for Samples

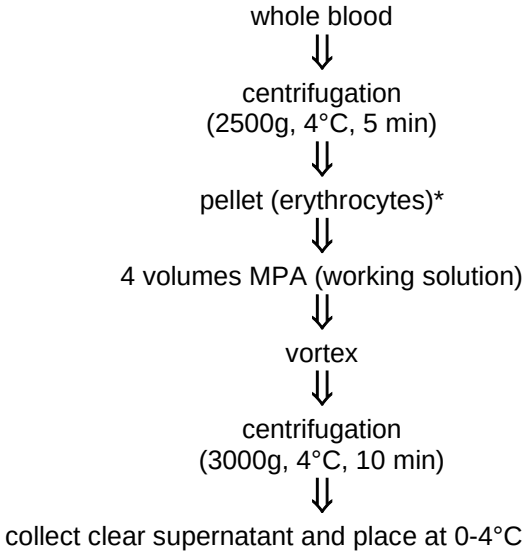
For each measurement, the reaction mixture is prepared as follows:

1. Take an initial volume (Vi) sample (4-60 µL).
2. Bring to 180 µL final volume with buffer (Volume of solution 3 = 180 µL - V_i).
3. Add 10 µL of solution R1 and thoroughly mix.
4. Add 10 µL of solution R2 and thoroughly mix.
5. Incubate at 25 ± 3°C for 10 minutes in the dark*.
6. Measure the final absorbance (A) at 400 nm.

*Samples are stable for 1 hour after 10-minute-incubation if they are kept in the dark.

Erythrocyte Lsate

1. Centrifuge a minimum of 500 µL of whole blood at 2500g at 4°C for 5 minutes.
2. Discard plasma supernatant. If not assayed immediately, store erythrocyte pellet at -70°C*.
3. Resuspend erythrocyte pellet in 4 volumes of MPA working solution, 0-4°C.
4. Thoroughly mix and centrifuge at 3000g at 4°C for 10 minutes.
5. Collect the upper clear aqueous layer and keep at 0-4°C for the assay (within 1 hour).



* Erythrocyte pellet can be stored at -70°C for 15 days.

Liver Homogenates

1. Wash tissue in 0.9% NaCl solution.
2. Blot tissue on paper and weigh.
3. Mince tissue in ice-cold MPA working solution.
4. Homogenize minced tissue.
5. Centrifuge homogenate at 3000g, 4°C for 10 minutes.
6. Collect the upper clear aqueous layer* and keep at 0-4°C for the assay (within 1 hour).

*Cloudy supernatant should be filtered through 0.2 µm filters.

Hepatocyte Lysates

1. Resuspend hepatocyte* pellet, from rats or mice, in 500 µL of ice-cold MPA working solution.
2. Homogenize cell suspension.
3. Centrifuge homogenate at 3000g, 4°C for 10 minutes.
4. Collect the upper clear aqeous layer and keep at 0-4°C for the assay (within 1 hour).

*Approximately 2.5-3.5 x 106 cells are used (5-8 mg of total protein).

Calculations

GSH Concentration

The calculation is based on the following equation:

$$[GSH] = \{(A-A_0)/(\varepsilon \times l)\} \times D$$

where:

[GSH]	is the initial glutathione concentration in the sample, expressed as molar concentration.
A and A_0	are the absorbances measured in the presence and in the absence of sample, respectively.
ε	is the apparent molar extinction coefficient of the product measured at 400 nm.
l	is the optical path (cm).
D	is the dilution factor of the sample.

Note:

- Do not add reagents R1 and R2 in reverse order.
- The temperature, (25 ± 3°C), should be kept constant throughout the experiment.
- The final reaction volume (0.2 mL) should not vary from one measurement to another.
- The optical density at 400 nm is proportional to glutathione concentration. It is stable for 60 minutes, provided that the reaction mixture is kept in the dark.

INTERFERENCES

Interferences are mainly due to the presence of proteins in samples. If the proteins are not precipitated in MPA working solution, the absorbance measured at 400 nm may not be stable.

Excess cysteine and alkylamines relative to glutathione concentration in samples gives an absorbance at 395 nm, which interferes with the glutathione assay. This interference is not usually seen in cell lysates or tissue homogenates, even if these samples contain very low levels of glutathione.

Oxidized glutathione (GSSG) is partially decomposed to GSH upon the addition of R2. Therefore, under conditions where the ratio of GSSG/GSH is equal or superior to 5%, GSSG significantly contributes to the absorbance reading at 400 nm.

Other mercaptans tested (Table 2) do not interfere significantly.

Table 2: Spectroscopic features of substitution products of various mercaptans with reagent R1 at 356 nm

Mercaptans	Apparent molar extinction coefficient 356 ($M^{-1}cm^{-1}$)	λ max (nm)
Glutathione	17400 ± 4 %	356
Mercaptopropionylglycine	17000 ± 4.8 %	353
Cysteinylglycine	15100 ± 7.5 %	350
L-Cysteine	24270 ± 2.5 %	352
L-Homocysteine	18000 ± 6.5 %	352
N-Acetyl-L-cysteine	16700 ± 1.7 %	358
DL-Penicillamine*	5600 ± 3.7 %	354
Dithiothreitol*	32200 ± 2.2 %	355
Mercaptosuccinic acid**	8800 ± 16 %	362
Captopril	17400 ± 0.7 %	359

*Reaction yield is not quantitative in 10 minutes when phosphate buffer pH 7.8 is used. With these mercaptans, a 0.1 M borate buffer pH 8.8 should be used instead of solution 3, in order to obtain a 100% reaction yield in 10 minutes.

**The two sulfhydryl groups of the molecule react with reagent R1, which results in approximately twice the color yield.

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1. Dolphin D., Poulson R. and Avramovic O., Eds. (1989), Glutathione: Chemical, Biochemical and Medical Aspects, Vols A & B, J. Wiley and Sons.
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3. Anderson M.E. (1989), Enzymatic and chemical methods for the determination of Glutathione; In: Glutathione: chemical, biochemical and medical aspects, Vol. A, Dolphin D., Poulson R. and Avramovic O. Eds., John Wiley and Sons, pp.339-365.
4. French Patent N° 9115868, Bioxytech® SA.

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