Total Glutathione (tGSH) Microplate Assay

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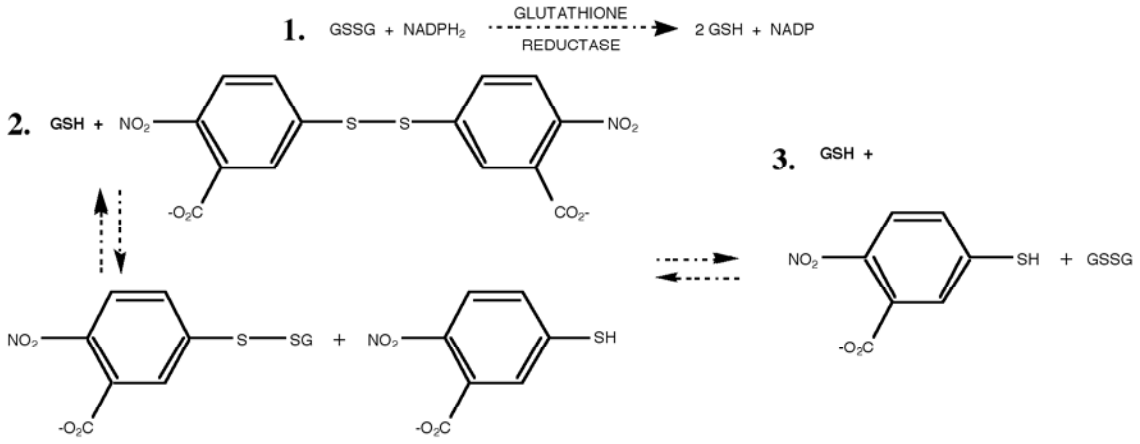
INTRODUCTION

Glutathione (γ-glutamylcysteinylglycine or GSH) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells. GSH is required as a coenzyme by a variety of enzymes including glutathione peroxidase, glutathione S-transferase and thioltransferase. GSH also plays a major role in drug metabolism, calcium metabolism, the γ-glutamyl cycle, cell membrane and blood platelet functions. 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 for the concentration of intracellular GSH generally range from 1 to 10 mM. Although many methods have been described for the assay of GSH, most of the reliable ones have been labor intensive.

PRINCIPLES OF PROCEDURE

This kit employs a kinetic enzymatic recycling assay based on the oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] to measure the total glutathione (tGSH) content of biological samples. The included glutathione standards or treated samples are added to the microtiter plate wells, followed by DTNB and glutathione reductase. Addition of β-$NADPH_2$ to the wells initiates the progressive reduction of DTNB by GSH, causing a color increase that is monitored at 405 nm. The rate of color change, followed over a 5 minute time period, is proportional to the tGSH concentration. Consequently, the concentration of tGSH in unknown samples may be determined by reference to the standard curve. GSH reacts with DTNB to produce a colored ion, which absorbs light at 405 nm, and a mixed disulphide. This disulphide reacts with further quantities of GSH present to liberate another ion and GSSG. GSSG is reduced enzymatically to GSH which then re-enters the cycle (see figure below). Since GSSG represents only a small percentage of total acid-solution free glutathione, the resulting values for tGSH (which encompasses both GSH and GSSG) are expressed in units of GSH equivalents.



MATERIALS PROVIDED

Component	Contents	Volume	Storage
Standard	Glutathione Standard (reduced form GSH); 2 mM	100 μL	-20°C
DTNB	5,5'-dithiobis(2-nitrobenzoic acid), lyophilized	1 vial	RT
β-NADPH$_2$	ß–Nicotinamide Adenine Dinucleotide Phosphate, reduced, lyophilized	1 vial	-20°C
Oxidoreductase	Glutathione Oxidoreductase	35 μL	-20°C
Assay Buffer	General buffer for diluting assay components	100 mL	4°C
MPA	5% Metaphosphoric Acid for sample deproteination and standard dilution	60 mL	4°C
Microplate	96-well microplate	1	RT

MATERIALS NEEDED BUT NOT PROVIDED

1. Plate reader with kinetic reading capability and 405 nm or 412 nm filters
2. Adjustable multi-channel and single-channel pipettes with disposable tips
3. Disposable glass test tubes
4. Vortex
5. Microcentrifuge tubes and 15 mL polypropylene centrifuge tubes

STORAGE

Store kit and all of its components according to the label specifications at all times.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as it may be detrimental to the assay.
3. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

PROCEDURAL NOTES

1. Reagents can be used immediately upon removal from refrigeration.
2. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

SAMPLE COLLECTION, PREPARATION, AND STORAGE

Whole Blood

1. Immediately after collection, the blood samples (≥ 0.25 mL) are deproteinated by the addition of four volumes of ice-cold 5% Metaphosphoric Acid (MPA).
2. Thoroughly mix and centrifuge at 13,000 x g at 4°C for 2 minutes. If the samples are not going to be processed further at this time, immediately store at –70°C. Process within 3 weeks.
3. Collect the upper aqueous layer and keep at 0-4°C for assay within 24 hours. If the experiment will not be performed within 24 hours, the MPA extract should be aliquoted into vials and stored at -20°C.
4. Just before analysis, the MPA extracts should be diluted 1:20 with Assay Buffer.

Erythrocyte Lysate

1. Immediately after collection, centrifuge at least 0.5 mL of whole blood at 3,000 x g at 4°C for 5 minutes.
2. Discard the plasma supernatant. If the sample will not be assayed immediately, store the erythrocyte pellet at –70°C and finish the processing within 3 weeks.
3. Resuspend the erythrocyte pellet in four volumes of ice-cold 5% MPA. This should be done on ice.
4. Thoroughly mix and leave on ice for 15 minutes. Centrifuge at 3,000 x g at 4°C for 10 minutes.
5. Collect the upper clear aqueous layer and keep at 4°C. Assay within 4 hours.

Hepatocyte Lysate

1. Resuspend the hepatocyte pellet (~10^6) in 0.5 mL of ice-cold 5% MPA solution.
2. Homogenize or sonicate the cell suspension and leave on ice for 5 minutes.
3. Centrifuge the homogenate at 3,000 x g at 4°C for 10 minutes.
4. Collect the upper clear aqueous layer and keep at 0-4°C. Test within 4 hours.
5. Just before analysis, the supernatant should be diluted 1:10 with assay buffer.

Tissue Extract

1. Wash tissue in 1x phosphate buffered saline (PBS), pH 7.2
2. Blot the tissue on filter paper.
3. Place the tissue (20 mg to 1 g) in 10 mL of ice-cold 5% MPA solution.
4. Homogenize thoroughly.
5. Centrifuge homogenate at 3,000 x g at 4°C for 10 minutes.
6. Collect the upper clear aqueous layer and keep at 0-4°C. Assay within 4 hours.

Human Urine and Saliva

1. Immediately after collecting the urine or saliva, add 4 volumes of ice-cold 5% MPA to the sample.
2. Thoroughly mix and centrifuge at 13,000 x g at 4°C for 2 minutes.
3. Collect the upper clear aqueous layer and keep at 0-4°C. Assay within 4 hours.

Cell Cultures in 96 well plates

1. After incubation, wash the cells (~10^4) gently with 1x phosphate buffered saline (PBS), pH 7.2.
2. Aspirate the washing solution and add 55-100 μL of 5% MPA to each well.
3. The culture plates are then frozen at –80°C and subsequently thawed at 37°C. This freeze/thaw cycle should be done twice and the plate left on ice for 15 minutes.
4. Remove 50 μL of the MPA extract from each well and transfer it to the assay microtiter plate using a multiple-channel pipette.
5. Transfer all the wells such that the plate configuration of the treated wells will be maintained in the assay microtiter plate. Leave space to run the GSH standard curve.

REAGENT PREPARATION

NOTE: The stability of reagents, once reconstituted, is greatly diminished. The kit should be used in its entirety within 2 hours of reagent reconstitution. During this time reagents should be stored as recommended in the following reconstitution instruction.

1. **NADPH:** Reconstitute contents of the vial with 500 μL Assay Buffer. Add this to 5.5 mL Assay Buffer and vortex. Leave on ice.
2. **Oxidoreductase:** Add 30 μL Oxidoreductase to 6 mL Assay Buffer. Leave on ice.
3. **DTNB:** Reconstitute contents of the vial with 500 μL Assay Buffer. Add this to 5.5 mL Assay Buffer and vortex. Leave at room temperature.

4. **GSH Working Stock:** Transfer 50 μL of Glutathione Standard to 950 μL of 5% MPA and vortex. Label as GSH Working Stock and leave on ice.

STANDARD CURVE PREPARATION

Prepare the GSH standards using the GSH Working Stock with a concentration range from 0.5 to 20 μM in 5% MPA or anywhere between 0-100 μM as needed. Prepare and label standards as follows in Table 1.

Table 1:

Standard	Concentration of GSH (μM)	Vol. of 5% MPA (μL)	Vol. of GSH Working Stock (μL)
B_0	0.0	1000	0
S_1	0.5	995	5
S_2	1.0	990	10
S_3	2.5	975	25
S_4	5.0	950	50
S_5	10	900	100
S_6	15	850	150
S_7	20	800	200

ASSAY PROCEDURE

1. Add 50 μL of each GSH standard or diluted or undiluted sample extract to each well.
2. Add 50 μL each of DTNB and Oxidoreductase solutions to each well.
3. Incubate the plate for 10 minutes at room temperature.
4. Add 50 μL of β-$NADPH_2$ solution to each well to start the reaction.
5. Within one minute after the addition of β-$NADPH_2$, read the plate using a kinetic program that can monitor the reaction at one minute intervals for 10 minutes. The rate of color change is monitored in the 405 - 412 nm range (depending on the filter wavelengths available on your plate reader). **Note:** If your plate reader does not have a kinetic reading function, then read the plate and record the OD value at 0 minutes and again at 10 minutes.

CALCULATIONS

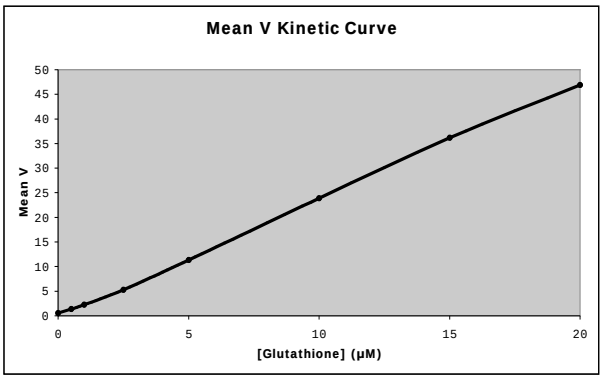
Plot the standard curve with Mean V versus GSH concentrations. The total GSH concentration of the samples can be calculated from the standard curve.

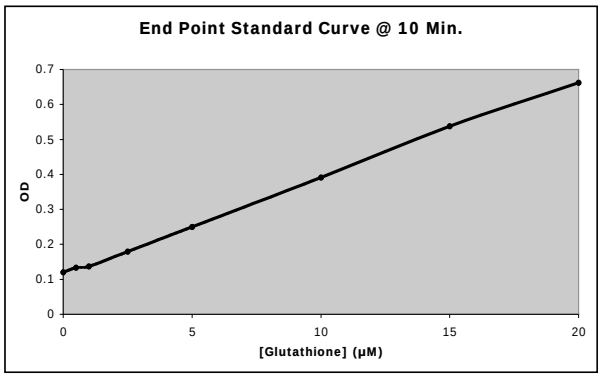
If a kinetic microplate reader is not available, the Mean V (the rate of color change) can be estimated by subtracting the OD value at 0 minutes from the OD value at 10 minutes and dividing the result by 10. That is:

$$\text{Mean V} = (\text{OD at 10 minutes} - \text{OD at 0 minutes}) / 10$$

The rate of color change represented as Mean V is proportional to the GSH standard concentrations.

Sample Curves:





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