Glutathione Assay (Colorimetric)

(Cat. #786-075, 786-076)

INTRODUCTION

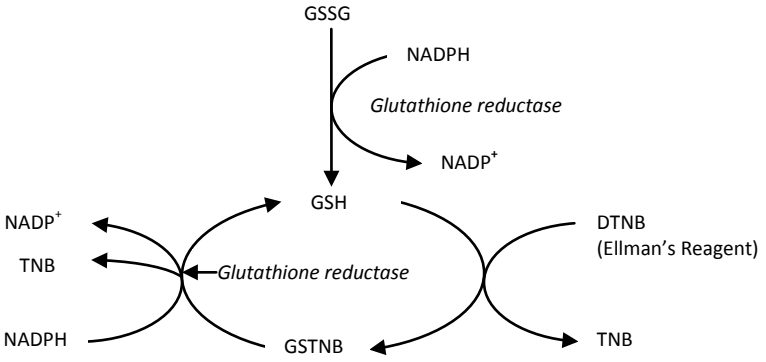
Oxidative stress is injurious to cells causing damage to DNA, proteins and lipids. It is caused by increased levels of reactive oxygen species (ROS) or/and decreased levels of antioxidants including reduced glutathione (GSH). Oxidative stress has been implicated to play a role in ageing and many pathological diseases, including cancer, diabetes, cardiovascular disorders, artherosclerosis, Parkinson's and Alzheimer's [1,2].

Reduced glutathione (L-γ-glutamyl-L-cysteinylglycine), a tripeptide, serves as a key antioxidant in animal, plant, fungi and bacteria by providing free thiol. Glutathione exist in reduced (GSH) and oxidized (GSSG; gluthathione disulphide) forms in cells and tissues, and the concentration of glutathione range from 0.5 to 10mM in animal cells[3]. The majority (90-95%) of glutathione exist in reduced form (GSH) in healthy cells. GSH provides reducing equivalents to antioxidant enzymes, hydroxyl radicals, ROS and is itself oxidized to GSSG; therefore GSH/GSSG ratio is critical indicator of the health of cell. During oxidative stress there is decrease in levels of GSH and increase in levels of GSSG and thus GSH/GSSG ratio decreases. GSH role as potential therapeutic agent for several diseases is a prominent area of research, due to its anti-oxidant effect.

Other functions of glutathione include synthesis of leucotrienes and prostaglandins, formation of mercapturates from electrophiles and storage and transport of cysteine. It is involved in many regulatory processes such as signal transduction and gene expression, DNA and protein synthesis and proteolysis, cell proliferation and apoptosis, cytokine and immune response, protein glutathionylation and mitochondrial function and integrity[3].

Glutathione colorimetric assay kit is designed to measure reduced glutathione (GSH), oxidized glutathione (GSSG) and total glutathione (GSH+GSSG) concentrations in wide range of samples such as, blood, plasma, serum, cultured cells and tissue. The assay involves carefully optimized enzymatic recycling method using glutathione reductase and Ellman's reagent (DTNB) [4]. Glutathione reductase reduces GSSG to GSH. DTNB (5-5'-dithiobis [2-nitrobenzoic acid]) reacts with GSH to form yellow color chromophore, 5-thionitrobenzoic acid (TNB) with absorbance maxima at 415 nm and GS-TNB. GS-TNB is further reduced to GSH and TNB by glutathione reductase, thus this enzymatic recycling of GSH enhances the sensitivity of the assay. The glutathione concentration of unknown sample is measured by measuring the absorbance at 415 nm and comparing it with standard curve for GSSG, which is plotted new every time when glutathione quantification is done.

Fig 1: Enzymatic recycling of reduced Glutathione



ITEM(S) SUPPLIED

Description	Cat. # 786-075 225 assays	Cat. # 786-076 450 assays
Oxidized Glutathione Standard (GSSG) [400 µM]	1 vial	2 vials
Glutathione Assay Buffer [5X]	20 ml	2 x 20 ml
Glutathione Reductase	50 µl	2 x 50 µl
NADPH	2 mg	2 x 2 mg
Ellman's Reagent	1 vial	2 vials
DMSO	1ml	1ml
Deproteination Reagent	2.5 g	2 x 2.5 g

STORAGE CONDITIONS

The kit is shipped on blue ice. Store NADPH and Oxidized Glutathione Standard at -20°C. Store all other components at 4°C. The kit components are stable for up to 1 year, when stored and used as recommended.

ADDITIONAL ITEMS REQUIRED

- PBS or other buffers required for sample processing
- 4-vinylpyridine (Cat. # 786-031)
- Clear Microtiter plates

PREPARATION BEFORE USE

1. Bring reagents to room temperature before performing assay and spin down the vials before reconstitution.

2. Dilute Glutathione Assay Buffer [5X] to 1X by diluting 1:4, i.e. add 1 ml Glutathione Assay Buffer [5X] to 4ml of deionized water.

3. Add125 µl 1X Glutathione Assay Buffer to each Glutathione Standard vial. Mix well and store in aliquots at -20°C.

4. Add 50 µl of deionized water to NADPH vial and mix well to dissolve. Make small aliquots and store at -20°C. 10 µl NADPH solution is suitable for 50 microwell assays.
   NOTE: *NADPH is light sensitive; therefore store in amber vials or protected from light.*

5. Add 0.5ml DMSO to the Ellman's Reagent vial and mix well. Make small aliquots and store at -20°C. 65 µl Ellman's Reagent solution is suitable for 50 microwell assays.
   NOTE: *Ellman's Reagent is light sensitive; therefore store in amber vials or protected from light.*

6. Deproteination solution: Prepare a 5% (w/v) Deproteination solution with supplied Deproteination Reagent in PBS or any other buffer. Use 50 mg Deproteination Reagent for every 1ml of solution required.
   NOTE: *Deproteination Reagent is light sensitive; therefore store in amber vials or protected from light. Prepare fresh each time and discard any unused solution.*

7. Prepare 1 M 4-vinylpyridine stock solution by adding 54 µl of 4-vinylpyridine to 446 µl ethanol and gently mix well. Discard the rest after use.
   NOTE: *4-Vinylpyridine is highly inflammable so it needs to be handled carefully and the working stock solution is prepared in a chemical fume hood.*
   NOTE: *4-Vinylpyridine is light sensitive. Make working stock in brown vials.*

PROTOCOL

Preparation of sample and Deproteination with 5% (w/v) Deproteination Reagent

Cell Lysate Preparation

1. Detach adherent cells by gentle trypsinization and centrifuge at 1000 x g for 5 min. Wash the cells with PBS and count them. For suspension cells, centrifuge at 1000 x g for 5 minutes and wash with PBS and count the cells.

2. Resuspend the pellet in 500 µl of cold 5% (w/v) Deproteination Reagent per 2-5 x 10^6 cells.

3. Homogenize or sonicate the cell suspension and store on ice for 5 minutes.

4. Transfer the cell suspension to a microcentrifuge vial and centrifuge at 12,000 - 14,000 x g for 5 minutes at 4°C. Collect the supernatant in a clean vial. Store on ice for immediate glutathione assay or freeze at -80°C for performing assay later.

Tissue Lysate Preparation

1. Remove the tissue and wash several times with cold isotonic saline (150 mM) or PBS. Weight the tissue before adding cold 5% (w/v) Deproteination Reagent.

2. Add 1 ml of ice cold 5% (w/v) Deproteination Reagent per 50mg of tissue and homogenize on ice.

3. Centrifuge the homogenate at 12, 000-14,000 x g for 10-15 minutes at 4°C.

4. Collect the supernatant in a new vial and store it on ice for immediate Glutathione assay or -80°C for performing assay later.

Erythrocyte and Whole Blood Lysate Preparation

1. Collect blood in tubes containing anticoagulant such heparin or sodium citrate. Centrifuge at 3,000 x g for 10-15 minutes at 4°C.

2. Discard the plasma and remove the white buffy coat on surface of erythrocytes.

3. Resuspend the erythrocyte pellet in four volumes of cold 5% (w/v) Deproteination Reagent and store on ice for 15 minutes.

4. Centrifuge the suspension at 12,000-14,000 x g for 10-15 minutes at 4°C.

5. Collect the supernatant. The supernatant can be stored on ice for immediate Glutathione assay or at -80°C for performing assay later.

6. For whole blood lysate preparation, add four volumes of cold 5% (w/v) Deproteination Reagent directly to the blood collected in tubes containing

anticoagulant. Mix thoroughly and store on ice for 15 minutes and do steps 4 to 5 as mentioned above.

Serum Preparation

1. Collect blood and allow it to clot for 30 min at room temperature.
2. Centrifuge the clotted blood at 2,000 x g for 15 minutes at 4°C. Collect the yellow serum in a new microcentrifuge vial and store on ice.
3. Add four volumes of cold 5% (w/v) Deproteination Reagent to serum and store on ice for 15 minutes.
4. Centrifuge the suspension at 12,000-14,000 x g for 10-15 minutes at 4°C.
5. Collect the supernatant. The supernatant can be stored on ice for immediate Glutathione assay or at -80°C for performing assay later.

Urine, Plasma, and Saliva Preparation

1. Collect urine, plasma or saliva and immediately add four volumes of 5% (w/v) Deproteination reagent. Mix thoroughly and store on ice for 15 minutes.
2. Centrifuge the suspension at 12,000-14,000 x g for 10-15 minutes at 4°C.
3. Collect the supernatant. The supernatant can be stored on ice for immediate Glutathione assay or at -80°C for performing assay later.

Working Assay Mixture preparation

- Immediately prior to use, prepare the Working Assay Mixture as shown in Table 1. Table 1 preparation is suitable for 48 reactions (100 µl/well)

Reagents	Volume
Glutathione Assay Buffer [1X]	5 ml
Glutathione Reductase	8.7 µl
NADPH solution	10 µl

Table1: Working assay mixture

NOTE: *NADPH is light sensitive, therefore make the assay buffer in a brown vial or cover the vial with aluminum foil.*

Ellman's Working Solution preparation

- Immediately prior to use, add 65 µl Ellman's Reagent stock solution to 2.5 ml of 1X Glutathione Assay Buffer to make a working solution. You require 50 µl working solution/ well.
  NOTE: *Ellman's Reagent is light sensitive, therefore make the working solution in a brown vial or cover the vial with aluminum foil.*

Assay protocol

1. Make dilutions of GSSG in microcentrifuge vials using 400 µM GSSG stock to achieve final concentration of 1 µM, 0.8 µM, 0.6 µM, 0.4 µM, 0.2 µM and 0.1 µM in 1X Glutathione Assay Buffer.
   Prepare 1 µM stock of Oxidized Glutathione Standard by adding 2.5 µl of 400 µM Oxidized Glutathione Standard solution to 1 ml of 1 X Glutathione Assay Buffer and mix well.

Tube	Oxidized Glutathione Standard [1µM]	1 X Glutathione Assay Buffer	Final Concentration of Oxidized Glutathione (µM)
A	-	200 µl	0
B	20 µl	180 µl	0.1
C	40 µl	160 µl	0.2
D	80 µl	120 µl	0.4
E	120 µl	80 µl	0.6
F	160 µl	40 µl	0.8
G	200 µl	-	1

NOTE: *The detection limit of this assay falls in 0.1-2.5 µM for GSSG and 0.2-5 µM for GSH, so different standard curves can be used.*
NOTE: *The standards should have the same concentration of Deproteination Reagent as the samples to ensure accurate estimations.*

2. Aliquot 50 µl GSSG standards into the wells performing in at least duplicate. (See format below).

3. Dilute the 5% Deproteination Solution in the samples to <0.5% by diluting the samples 1:10 with 1X Glutathione Assay Buffer.
   NOTE: *Lower than 0.5% Deproteination Reagent in sample is acceptable for the assay, however >0.5% Deproteination Reagent is not recommended as it may interfere with the assay. The standards should have the same concentration of Deproteination Reagent as the samples to ensure accurate estimations.*

4. Aliquot 50 µl samples into the wells performing in at least duplicate. (See format below).

5. Add 100 µl of freshly prepared working assay mixture per well.

6. Incubate the plate at room temperature for 5 minutes.

7. Rapidly add 50 µl of freshly prepared Ellman's Reagent working stock solution per well and mix several times by pipetting up and down.

8. Cover the plate with aluminum foil or incubate plate in dark on shaker until absorbance is checked. For kinetic method absorbance at 0 minute is also recorded

9. Glutathione concentration can be determined by endpoint method or by kinetic method

   End point method: Read the plate at 405-415 nm, 25 minutes after addition of Ellman's Reagent.

   Kinetic method: Read the plate at 405-415 nm at 5 minutes interval after addition of substrate for 30 minutes

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	S1	S1								
B	0.1 µM	0.1 µM	S2	S2								
C	0.2 µM	0.2 µM	S3	S3								
D	0.4 µM	0.4 µM	S4	S4								
E	0.6µM	0.6µM	S5	S5								
F	0.8 µM	0.8 µM										
G	1 µM	1 µM										
H												

Table 2: Assay format: A1-G1 and A2-G2 for standard for GSSG. Samples are checked for glutathione concentration in duplicate set. The above assay protocol is for total glutathione estimation (GSH + GSSG). Another set of 24 wells can be used for oxidized glutathione (GSSG) detection. The above assay format is just an example. It can be changed as per requirement.

Oxidized Glutathione concentration measurement using GSH derivatizing reagent 4-vinylpyridine

- To exclusively quantify GSSG in sample, the samples and the standards need to be treated with 4-vinylpyridine.
  NOTE: *4-vinylpyridine blocks any free thiols present in the solution. Even though standard graph is made with only GSSG stock solution still the standards need to be treated with 4-vinylpyridine as in interferes with glutathione assay and thus the effect is equally subtracted when same 4-vinyl pyridine treatment is done to the standards as well.*

- Add 1 µl 1 M 4-vinylpyridine to 50 µl GSSG stock solution [400 µM] and add 1 µl 1 M 4-vinylpyridine per 50 µl 1 X glutathione assay buffer. Similarly treat the sample with 4-vinylpyridine. Incubate the tubes at RT for 1 hr.

- Make dilutions of GSSG in microcentrifuge vials using 4-vinylpyridine treated 400 µM GSSG stock to achieve final concentration of 1 µM, 0.8 µM, 0.6 µM, 0.4 µM, 0.2 µM and 0.1 µM in 1 X glutathione assay buffer.
  ***NOTE**: As mentioned in assay protocol, care should be taken that concentration of 5% Deproteination Solution is brought lower than equal to 0.5% with 1 X glutathione assay buffer. Also standards for glutathione should have same concentration of Deproteination Solution. 4-vinylpyridine treated sample needs to be diluted with 1 X Glutathione Assay Buffer similarly like GSSG stock solution.*

- Take 2.5 µl of 4-vinylpyridine treated 1 X Glutathione Assay Buffer and add this to 1 ml of 1 X glutathione assay and mix well and use this for blank.

- Repeat steps 2-8 mentioned in assay protocol to measure oxidized glutathione concentration

GSH concentration = Total Glutathione – oxidized glutathione (GSSG)

Reduced glutathione concentration is calculated by subtracting oxidized glutathione concentration from total glutathione concentration

Glutathione measurement by end-point method

1. Measure the absorbance of samples and standard at 415 nm after 25 minutes

2. Calculate the average absorbance of standard and samples. Subtract the blank absorbance from sample and standards to get the corrected value for absorbance.

3. Plot the corrected absorbance as function of oxidized glutathione concentration Fig 2 and Fig 3.

4. Calculate the total glutathione concentration for sample

   $$\text{Total glutathione (GSSG +GSH)} = \frac{[\text{OD at } 415 - (\text{y-intercept})]}{\text{Slope}} \times \text{sample dilution} \times 2^{*}$$

   * The equation is multiplied by 2 as 1 GSSG= 2 GSH

5. Similarly calculate oxidized glutathione (GSSG) concentration from 4- vinyl pyridine treated glutathione standards and sample

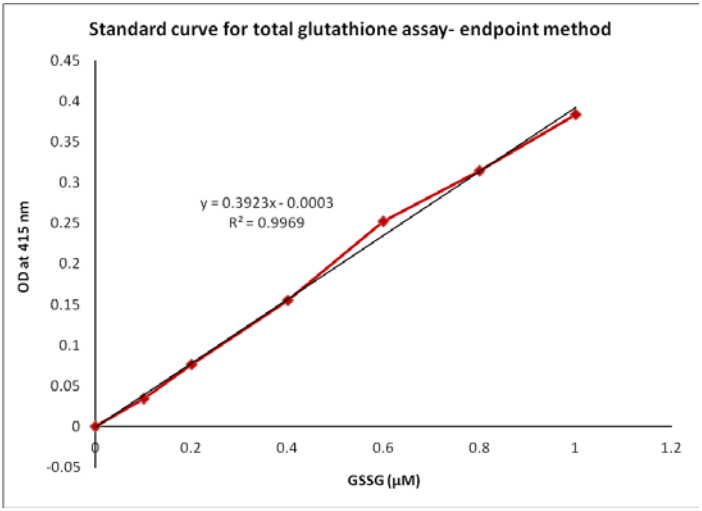


Fig 3: Standard curve for total glutathione assay by end-point method

Glutathione measurement by kinetic method

1. Measure the absorbance of standards and samples after every 5 minutes starting with 0 minute for 30 minutes

2. Plot the average absorbance values of each standard and sample as a function of time (Fig. 4) and determine the slope (OD at 415/min) for each curve.

3. Plot rate of increase in absorbance at 415 per minute (ΔOD 415/min) as function of oxidized glutathione concentration (Fig. 5).

4. Calculate the total glutathione concentration for sample

$$\text{Total glutathione (GSSG +GSH)} = \frac{[\Delta\text{OD at } 415/\text{min} - (\text{y-intercept})]}{\text{Slope}} \times \text{sample dilution} \times 2^{*}$$

* The equation is multiplied by 2 as 1 GSSG= 2 GSH

5. Similarly calculate oxidized glutathione (GSSG) concentration from 4- vinyl pyridine treated glutathione standards and sample

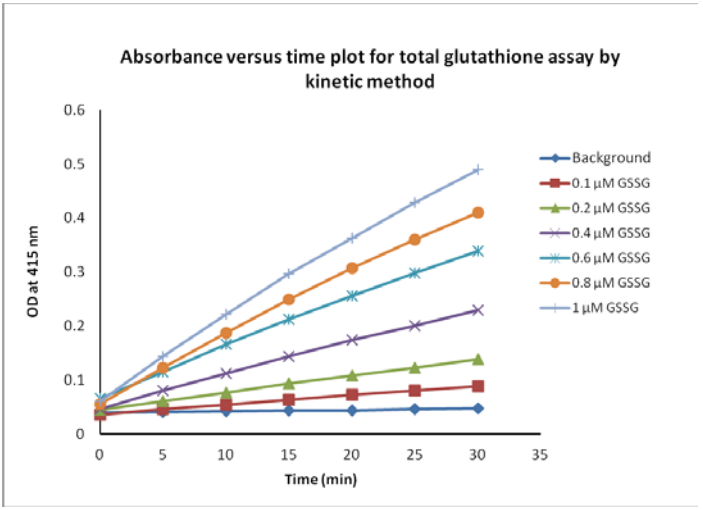


Fig 4: Absorbance versus time plot for total glutathione assay by kinetic method

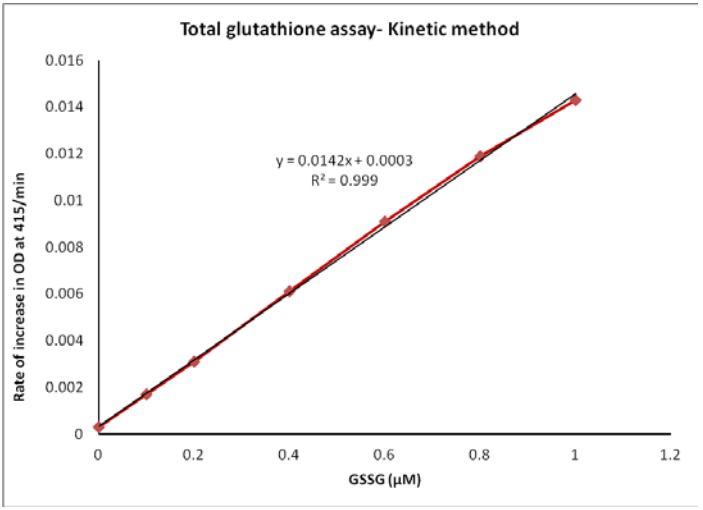


Fig 5: Total glutathione assay by kinetic method

Effect of 4-vinyl pyridine on reduced glutathione (GSH)

4-vinylpyridine is a better derivatizing reagent for free thiols like GSH when compared to N-ethyl-maleimide (NEM) as N-ethyl-maleimide is a potent inhibitor of glutathione reductase. 4-vinylpyridine treatment removes all the free thiols present in the sample leaving only GSSG which can be quantified in the same way as total glutathione using Ellman's Reagent.

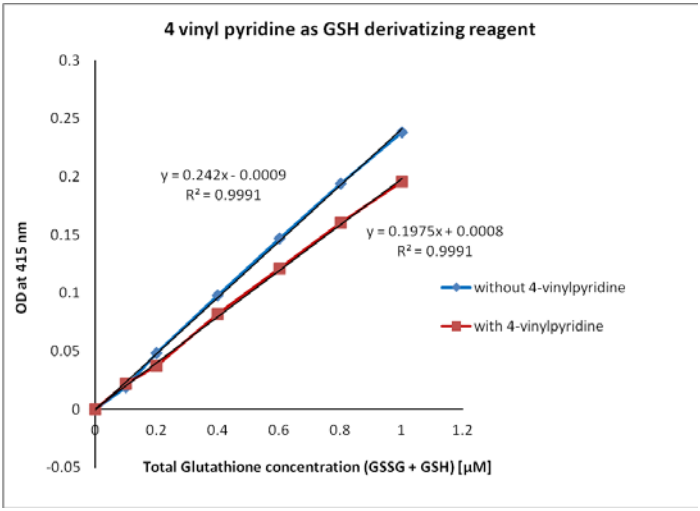


Fig 6: Effect of 4-vinyl pyridine on reduced glutathione (GSH). Standard was prepared from stock solution (400 µM) of mixture of GSH and GSSG

INTERFERENCES

Thiols like β–mercaptoethanol, dithiothreitol and reducing agents like ascorbic acid and cysteine interfere with the glutathione assay. Thiol alkylating agents such as N-ethylmaleimide also interfere with the assay.

TROUBLESHOOTING

Issue	Suggested reason	Possible solution
Inconsistent values in duplicate or triplicates	1.Poor pipetting 2. Bubbles in well	1.Pipette carefully 2.Tap the side of plate gently to remove air bubbles
There is no development of color	One or more of the constituents of working assay mixture missing or the standards and samples not added to the well	Ensure all the constituents of assay mixture are present. Also the standards and sample added to wells and substrate is added
Standard curve is not linear	GSSG concentration to high	Lower the concentrations of GSSG standards used for standard curve
No color in the sample above the background	Sample is too diluted	Concentrate the sample by lyophilization and reconstitute in small volume of 1 x glutathione assay buffer
The sample has absorbance value higher than the highest point of the standard curve	Sample is too concentrated	Dilute the sample and reassay

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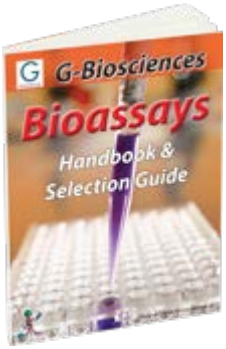

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