*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® GSH/GSSG-412™

Colorimetric Determination of Reduced and Oxidized Glutathione
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21040

INTRODUCTION

The Analyte

Reduced glutathione (GSH), a tripeptide (γ-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol. During this process GSH becomes oxidized glutathione (GSSG). The GSSG is then recycled into GSH by gutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH). When mammalian cells are exposed to increased oxidative stress, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation. Measurement of the GSSG level or determination of the GSH/GSSG ratio, is a useful indicator of oxidative stress and can be used to monitor the effectiveness of antioxidant intervention strategies.

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$
$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

PRINCIPLES OF THE PROCEDURE

The accurate measurement of GSSG levels has proved very difficult due to the low amount of GSSG in tissues and because of the absence of effective methods to prevent oxidation of GSH to GSSG during sample preparation. To measure GSSG in tissues, Guntherberg and Rost (2) first introduced N-ethylmaleimide (NEM) to eliminate the GSH. Although NEM can react with GSH to form a stable complex and prevent the participation of the reduced form in the enzymatic assay, NEM also inhibits GR. For this reason, Griffith (3) first introduced 2-vinylpyridine (2-VP), which does not inhibit GR significantly, to derivatize GSH. However, the 2-VP reaction is relatively slow and the reagent has little solubility in an aqueous medium.

The GSH/GSSG-412 assay uses the thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate[1] (M2VP) at a level that rapidly scavenges GSH but does not interfere with the GR assay.

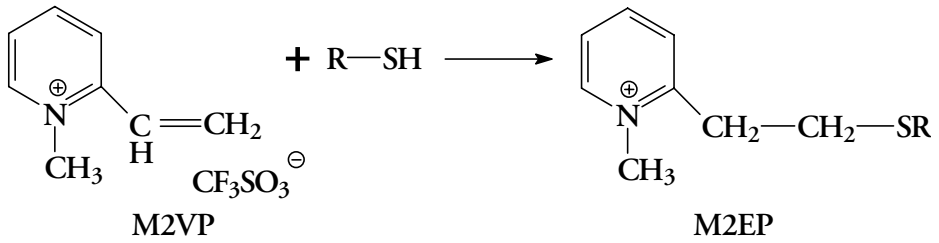


Figure 1. Reaction of M2VP with a thiol to form 1-Methyl-2-(2-thioethyl)-pyidinium salt.

[1] US Patent 5,543,298

2-VP at 10 mM, usually takes 60 minutes to remove 70% of the GSH in the sample during which time oxidation of GSH may occur, resulting in significant overestimation of the GSSG concentration. Using M2VP, complete scavenging of GSH is accomplished in less than one minute.

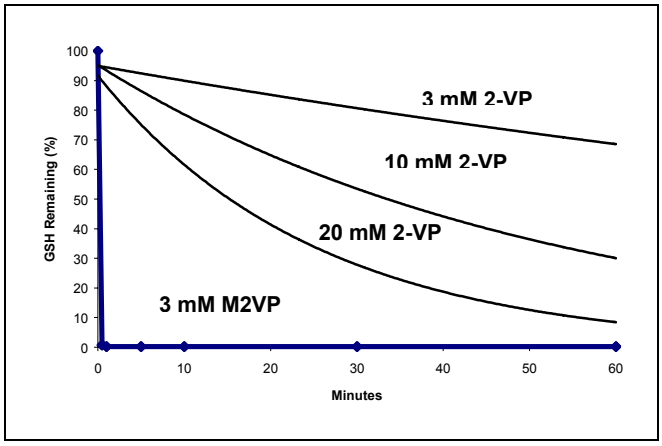
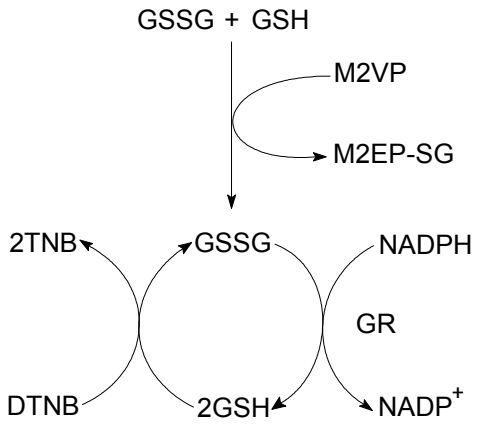


Figure 2. :GSH was added to three concentrations of 2-VP and M2VP at 3 mM. The remaining GSH was determined using the GSH/GSSG-412 Assay.

In 1969, Tietze (1) first introduced an enzymatic method for quantitative determination of amounts of total[2] (reduced and oxidized or GSH_t) glutathione. The method employs Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB), which reacts with GSH to form a spectrophotometrically detectable product at 412 nm. GSSG can be determined by the reduction of GSSG to GSH, which is then determined by the reaction with Ellman's reagent. In brief, the Tietze method (1) utilizes the change in color development during the reaction, and the reaction rate is proportional to the GSH and GSSG concentrations.



[2] The definition of total GSH in this context does not include other mixed disulfides of glutathione such as glutathione-cysteine.

REAGENTS

Materials Provided (for 200 tests)

- Assay Buffer — $Na{\cdot}PO_4$ with EDTA, dry powder.
- GSSG Buffer — $Na{\cdot}PO_4$ with EDTA, 150 mL.
- Enzyme — Glutathione reductase (GR) in $Na{\cdot}PO_4$ with EDTA, 40 mL.
- NADPH — β–Nicotinamide adenine dinucleotide phosphate with Tris base and mannitol, 6 vials lyophilized powder.
- Scavenger — 1-Methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) in HCl, 2 mL.
- Chromogen — 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) in $Na{\cdot}PO_4$ with EDTA, with ethanol, 2 x 20 mL.
- Standards — GSSG in KPO_4 buffer with EDTA, 2.0 mL each. Each GSSG molecule is equivalent to two GSH molecules; therefore, the values are expressed as µM GSH:

	GSSG, µM	GSH, µM
1	0.000	0.00
2	0.050	0.10
3	0.125	0.25
4	0.250	0.50
5	0.750	1.50
6	1.500	3.00

Items Required But Not Provided

- Spectrophotometer
- Centrifuge 1000 x *g* or greater
- Spectrophotometric cuvettes with a 1 cm optical path length (glass or polystyrene).
- Disposable tubes and stoppers (glass or polypropylene).
- Pipettes, preferably adjustable cabable of accurately pipetting 10, 50, 100, 200, 700 and 3000 µL.
- Balance
- Metaphosphoric acid (Sigma M-5043)

Reagent Storage and Handling

- When not in use, place the bottles at 4°C. Do not allow the capped reagent bottles to sit at room temperature for long periods of time.
- Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

- NADPH: Just prior to use, reconstitute the lyophilized NADPH Reagent with 7.5 mL of Assay Buffer. The reconstituted NADPH Reagent is stable for 6 hours at room temperature.

- Assay Buffer: Reconstitute the dry powder with 650 mL of deionized water. The reconstituted reagent is stable at 4^{o}C for the life of the kit.

- 5% Metaphosphoric Acid: Prepare fresh daily. Weigh 1 gram MPA and dissolve in 20 mL deionized water.
- MPA and NADPH reagents are intended for same day use following reconstitution.

Preparing GSSG Standard

Standards are ready to use.

Sample Preparation

The sample preparation for whole blood is described below. Please read the appropriate **NOTES** sections for further information regarding the whole blood assay as well as procedural guidelines for other sample types.

GSSG Sample

1. Add 10 µL M2VP to a microcentrifuge tube (recommended).
2. Carefully add 100 µL whole blood to the bottom of the centrifuge tube.
3. Mix gently.
4. Freeze the sample at –70°C. (Sample is stable for at least 30 days at –70°C).
5. Thaw the sample and immediately mix, incubate at room temperature for 2-10 minutes.
6. Add 290 µL cold 5% MPA to the tube (1/4 dilution of original sample).
7. Vortex the sample for 15-20 seconds.
8. Centrifuge at 1000 x *g* or greater for 10 minutes.
9. Add 50 µL MPA extract to 700 µL GSSG buffer (1/15 dilution of the acid extract).
10. Place the diluted extract on ice until use (Final sample dilution is 1/60).

GSSG Blank

1. Add 50 µL MPA to 700 µL GSSG buffer (1/15 dilution of the acid extract).
2. Place the diluted MPA on ice until use (Final sample dilution is 1/60).

GSH Sample

1. Carefully add 50 µL of whole blood to the bottom of a microcentrifuge tube (recommended).
2. Freeze the sample at –70°C. (Sample is stable for at least 30 days at –70°C).
3. Thaw the sample and immediately mix.
4. Add 350 µL cold 5% MPA to the tube (1/8 dilution of original sample).
5. Vortex the sample for 15-20 seconds.
6. Centrifuge at 1000 x *g* or greater for 10 minutes.
7. Add 50 µL MPA extract to 3 mL Assay Buffer (1/61 dilution of the acid extract).
8. Place diluted extract on ice until use (Final sample dilution is 1/488).

Assay

1. Add 200 µL of standards, blank or samples to the cuvettes.
2. Add 200 µL of Chromogen to each cuvette.
3. Add 200 µL of Enzyme to each cuvette.
4. Mix and incubate at room temperature for 5 minutes.
5. Add 200 µL of NADPH to each cuvette.
6. Record the change of absorbance at 412 nm for 3 min.

CALCULATIONS

The calculation of the GSH and GSSG concentrations and the GSH/GSSG ratio requires four steps: 1) Determination of the reaction rate, 2) Construction of calibration curves, 3) Calculation of the analyte concentrations, and 4) Calculation of the GSH/GSSG ratio.

Rate Determination

The change in absorbance at 412 nm is a linear function of the GSH concentration in the reaction mixture, is described by the following equation of a line:

$$A_{412} = \text{slope} \times \text{Minutes} + \text{intercept}$$

where the slope of the regression equation is equal to the rate. Note that the intercepts for these rate curves are ignored because they are dependent on the DTNB background and the time interval between the addition of the NADPH (reaction start) and the first recorded A_{412} measurement.

In the examples below, linear regression gave the following equation of the line for the GSH_t (**Figure 3**) and the GSSG (**Figure 4**) samples:

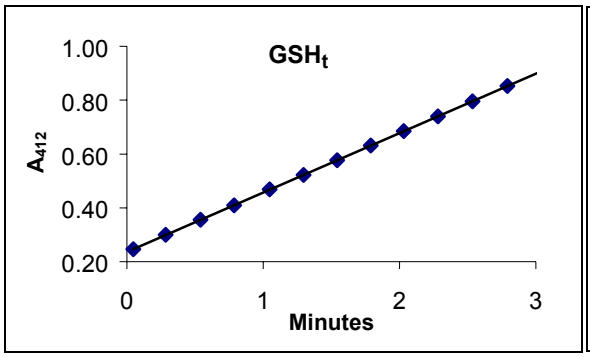


Figure 3. Reaction rate for an untreated sample. The rate is proportional to the concentration of GSH_t.

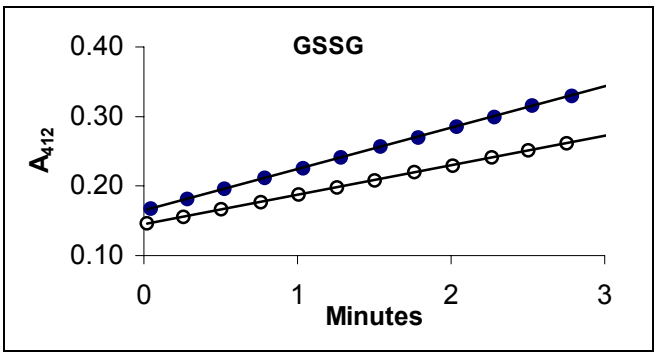


Figure 4. Reaction rate for a M2VP treated sample (●) and the GSSG Blank (o). The rate is proportional to the concentration of GSSG.

GSH_t: A_{412} = 0.2209 x Minutes + 0.2363 with an r^2 value of 1.0000. Therefore, the rate for the GSH_t sample is 0.2209 A_{412}/min.

GSSG: A_{412} = 0.05938 x Minutes + 0.1651 with an r^2 value of 0.9999. Therefore, the rate for the GSSG sample is 0.05938 A_{412}/min.

GSSG BLANK: A_{412} = 0.04238 x Minutes + 0.1454 with an r^2 value of 0.9999. Therefore, the rate for the GSSG Blank is 0.04238 A_{412}/min.

Calibration Curves

The GSH/GSSG-412 assay uses a six-point standard curve for both GSH_t and GSSG determinations. The Net Rate is the difference between the rate at each concentration of GSH and the Blank rate.

Table 1. A typical 6 point calibration of the GSH/GSSG-412 Assay.

µM GSH	A_{412}/min	Net Rate
0.00	0.0423	0
0.10	0.0571	0.0148
0.25	0.0789	0.0366
0.50	0.1161	0.0738
1.50	0.2605	0.2182
3.00	0.4762	0.4339

Because the concentration of GSSG is much lower in the reaction mixture compared to GSH_t, it is recommended that selected data ranges from the calibration curve be plotted separately. For GSH_t, perform linear regression on a three-point curve using the 0, 1.50 and 3.00 µM GSH data points, see **Figure 5**. In the case of GSSG, use the 0, 0.10, 0.25, and 0.50 µM GSH data points, see **Figure 6**.

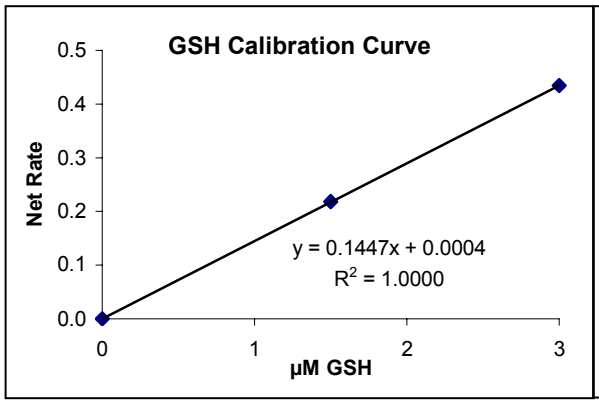


Figure 5. A three point calibration curve of A_{412}/min vs. µM GSH used to determine the concentration of GSH_t.

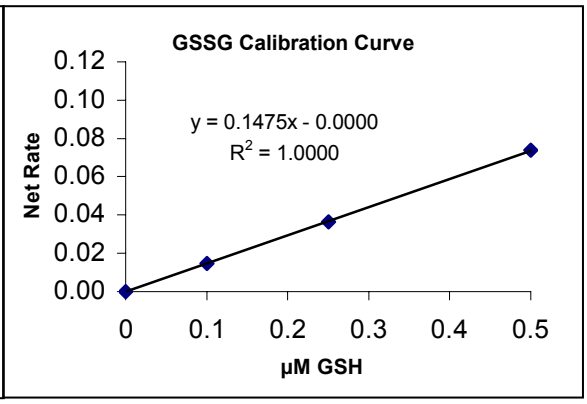


Figure 6. A four point calibration curve of A_{412}/min vs. µM GSH used to determine the concentration of GSSG.

GSH_t and GSSG Concentrations

The general form of the regression equation describing the calibration curve is:

Net Rate = slope x GSH + intercept

Therefore, to calculate the analyte concentration from the GSH calibration curve:

$$\text{GSH} = \frac{\text{Net Rate} - \text{intercept}}{\text{Slope}} \bullet \text{dilution factor}$$

For example, from **Figure 3**, the net rate of change for the GSH_t sample is 0.2209 – 0.0423 or 0.1786 A_{412}/min. Using the calibration curve parameters from **Figure 5**, the GSH_t can be calculated as follows:

$$\text{GSH}_t = \frac{0.1786 - 0.0004}{0.1447} \bullet 488 = 601.0\,\mu\text{M}$$

Precision

The precision of the GSH/GSSG-412 assay was determined using a modification of the NCCLS EP5-T2 guideline. A sample containing a high and low concentration of GSSG was assayed twice per day for 13 days. At the same time, the GSH_t and GSSG were determined from whole blood samples stored at –70°C. Each precision parameter was expressed as the mean, standard deviation, and coefficient of variation of the net rate were observed in each sample set.

	GSSG in Buffer		**Whole blood**	
	High	**Low**	**GSH**	**GSSG**
Mean N = 26	0.4194	0.0427	0.2938	0.00490
Intra-assay standard deviation	0.0025	0.0015	0.0028	0.00031
Inter-assay standard deviation	0.0119	0.0013	0.0091	0.00037
Total standard deviation	0.0120	0.0016	0.0093	0.00043
Intra-assay (%CV)	**0.59**	**3.50**	**0.96**	**6.45**
Inter-assay (%CV)	**2.84**	**2.96**	**3.11**	**7.61**
Total precision (%CV)	**2.87**	**3.86**	**3.18**	**8.86**

Sensitivity

The enzymatic assay is for total GSH, including both GSH and GSSG. The Lower Limit of Detection (LLD) is a measure of the lowest GSSG concentration based on the differences of blank rates, which will be defined as 3.29 standard deviations above zero.

Number of data points	14
Mean A412/min	0.0427
Standard deviation (s)	0.000802
3.29 x *s*	0.002639
LLD in µM reaction mixture	0.009
LLD in µM original sample	0.54

Specificity

The GSH analogues were tested in buffer solution and the interference was calculated by comparing with the slope of the GSSG standard. The values are expressed as a percent of the GSSG standard. All tested analogues have no interfering effect on the GSH/GSSG-412 assay.

Analogues	µM	Net Rate at A412	µM GSH	% of GSH
GSH	2.5	0.3653	2.50	100
Cysteine	2.5	0.0006	0.0041	0.16
Cystine	2.5	0.0005	0.0034	0.14
N-acetylcysteine	2.5	-0.0014	-0.0096	-0.38
Captopril	2.5	-0.0011	-0.0075	-0.30
Cysteinylglycine	2.5	0.0031	0.0212	0.85
Dithiothreitol	2.5	0.0007	0.0048	0.19
Ergothioneine	2.5	0.0002	0.0014	0.05
Homocysteine	2.5	0.0011	0.0075	0.30
Penicillamine	2.5	-0.0011	-0.0075	-0.30
Mercaptosucininc acid	2.5	0.0004	0.0027	0.11
Mercaptopropinylglycine	2.5	0.0030	0.0205	0.82
Acetaminophen	2.5	0.0008	0.0055	0.22
Ibuprofen	2.5	-0.0004	-0.0027	-0.11

Sample Correlation With Reference Assay

A comparison between the enzymatic assay and the HPLC-electrochemical detection assay has been reported by Richie *et al*. (4). They reported that there was no significant difference between these two methods in the measurement of human whole blood GSH.

NOTES

Interference

There are no known interferents for the GSH/GSSG-412 Assay.

Sample Preparation

Tissues

Tissues have not been tested with the GSH/GSSG-412 Assay. The following issues need to be considered when preparing tissue samples for analysis:

- GSH oxidation *in vitro* likely occurs rapidly in disrupted tissues. The M2VP reagent should be added as rapidly as possible.
- GSSG diffusion from the cell may result in underestimating the GSSG. This is likely to be only an issue with extensively washed cells.
- γ-Glutamyltranspeptidase will metabolize GSH. This membrane bound enzyme is especially high in kidney, pancreas, ciliary body, choroids plexus, intestinal epithelia, bile duct cells, lymphoid cells and many tumor cells (5).

Whole Blood

Pipetting. Whole blood is difficult to pipette with precision. Using positive displacement pipetting techniques will improve the precision of the GSH and GSSG assays with whole blood.

Freezing Step. The freezing step serves to lyse the red blood cell and maximize the concentration of GSSG in the sample.

Frozen Samples. Blood samples that have been frozen without prior treatment with the Scavenging Reagent are not suitable for the GSSG assay.

GSH Linearity. Because GSH is at high concentrations in whole blood, approximately 1 mM of the whole blood sample should be diluted at least 244 times in order to maintain linearity of the reaction rate.

Sample Stability. Glutathione and oxidized glutathione are relatively stable in intact "resting" cells for up to 24 hr at 4°C, **Figure 7**. The stability of "elevated" GSSG in the intact red blood cell has not been determined. It is recommended that blood samples be treated with M2VP as soon as possible and frozen immediately.

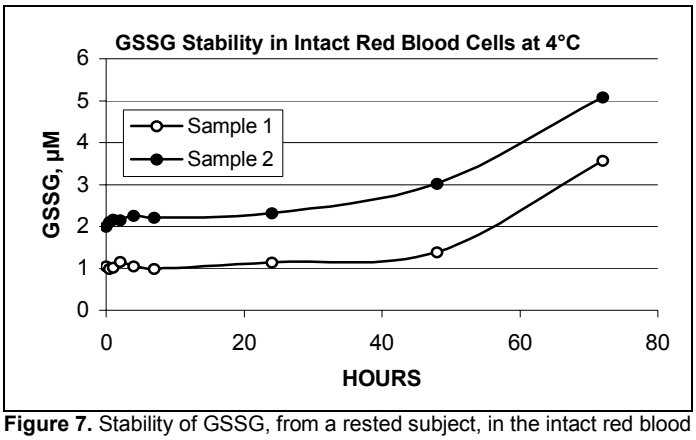


Figure 7. Stability of GSSG, from a rested subject, in the intact red blood cell at 4°C. (GSH-159).

Upon disruption of the cell, GSH is rapidly oxidized as shown in **Figure 8**. To minimize the *in vitro* oxidation of GSH to GSSG, the Scavenger Reagent should be added to the sample prior to lysis or homogenization.

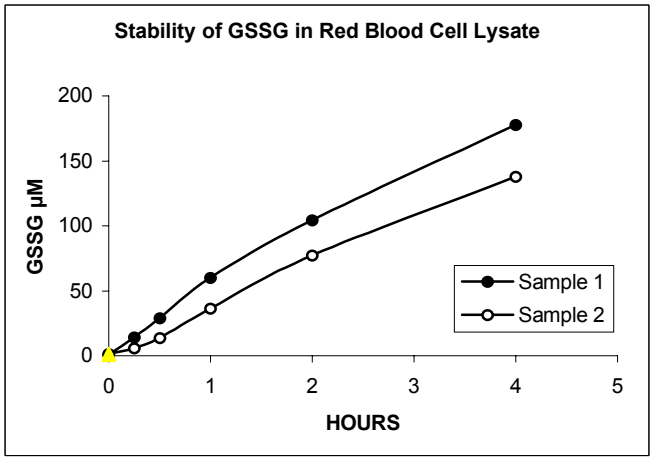


Figure 8. Stability of GSSG in the red blood cell lysate at 4°C. (GSH-161)

Normal Plasma

GSSG in normal resting plasma is at or below the lowest level of detection for the assay.

Urine

GSSG is not detectable in urine.

Limitations

This assay has been validated only for whole blood samples containing EDTA as the anticoagulant.

Assay Performance

The rate curves must be linear. If not, generally this means the GSH concentration is too high. Dilute the sample and re-run the assay.

Calibration curves must be linear.

REFERENCES

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