Northwest
Life Science Specialties, LLC

5131 NE 94th Avenue, Suite 201
Vancouver, WA 98662
Phone 360-449-3091 or Toll Free: 888-449-3091
Fax 360-449-3092
E-mail: nwsales@nwlifescience.com

Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Glutathione Assay

Product NWK-GSH01
For Research Use Only

Simple assay kit for quantitative measurement of glutathione in biological samples such as whole blood, tissue homogenates and cell lysates.

nwkgsh01rev010307

Table of Contents

End-User Notes:

nwkgsh01rev010307

Procedure Checklist

__ Process the sample and place on ice

__ Prepare 5% MPA and place on ice

__ Prepare Calibrators and place on ice

__ Bring GSH reagents to ambient temperature

__ Setup microplate reader
Wavelength = 405 nm
Mode = Kinetic
Reaction Time = 3 Minutes
Read Interval = 15-20 seconds (9-12 data points)

__ Deproteinate samples 50:100; sample to MPA

__ Centrifuge sample for 1 minute at 10,000 x *g*

__ Dilute samples 25:500; supernatant to Assay Buffer and place on ice

__ Reconstitute NADPH Reagent with NADPH Reagent Diluent

__ Add 50 µL calibrators, controls and diluted samples to appropriate wells of microplate

__ Add 50 µL DTNB Reagent to all wells

__ Add 50 µL GR Reagent to all wells

__ Incubate 2-3 minutes at ambient temperature

__ Add 50 µL NADPH Reagent

__ Place microplate in plate reader and begin 405 nm measurements

__ Calculate results

__ Return reagents to 2-8°C.

Introduction:

Glutathione (GSH, γ -glutamylcysteinylglycine), the primary non-protein sulfhydryl in aerobic organisms is synthesized in most cells. The ubiquitous tripeptide is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH.

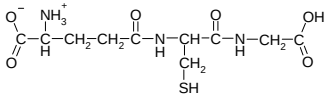

Figure 1.
Structure of GSH

In addition to donating an electron during the reduction of hydroperoxides to the respective alcohols (or water in the case of hydrogen peroxide), GSH functions as a co-substrate in the metabolism of xenobiotics catalyzed by glutathione S-transferases. It is also a co-factor for several metabolic Enzymes, is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation.

Intended Use:

The NWLSS™ Glutathione Assay is used to measure the concentration of total GSH (reduced and oxidized) in a variety of animal and plant samples.

Test Principle:

The NWLSS™ Glutathione Assay is a modification of the method first described by Tietze.[2] The general thiol reagent, 5-5′-dithiobis[2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with GSH to form the 412 nm chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB. The GS-TNB is subsequently reduced by glutathione reductase and β-nicotinamide adenine dinucleotide phosphate (NADPH), releasing a second TMB molecule and recycling GSH; thus amplifying the response. Any oxidized GSH (GSSG) initially present in the reaction mixture or formed from the mixed disulfide reaction of GSH with GS-TNB is rapidly reduced to GSH.

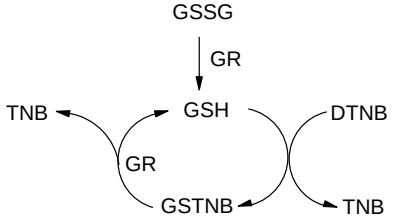

Figure 2.
GSH Recycling Pathway

General Specifications:

Format:: 2 X 96 wells

Number of tests: Triplicate = 58
Duplicate = 90

Specificity: Total Glutathione (GSH plus GSSG)

Sensitivity: LLD = 0.1 µM in the reaction
(Calculated as 3.29 standard deviations from zero).

Kit Contents:

NADPH: β-Nicotinamide adenine dinucleotide phosphate, reduced; 2 vials powder.

NADPH Diluent: Buffer with stabilizer; 2 x 6 mL vials.

DTNB: 5-5′-Dithiobis(2-nitrobenzoic acid) in phosphate buffer with EDTA; 1 x 11 mL.

GR Enzyme: Glutathione reductase in Assay Buffer with protein stabilizer; 1 x 11 mL.

Calibrators: Glutathione disulfide in 1 mM HCl, 400 µM GSH equivalents; 1 x 0.5 mL.

Assay Buffer: Phosphate buffer with EDTA, pH 7.6; 1 x 125 mL

Required Materials Not Provided:

Pipettes capable of transferring 25, 50, 100 and 500 µL volumes.
A multi-channel or repeater pipette (recommended)
Metaphosphoric acid , (Sigma 239275 or equivalent).
Deionized water
Polypropylene microcentrifuge tubes or equivalent
Stir bar
Beaker or flask 25-50 mL

Required Instrumentation:

Microplate reader with kinetics capability at 405 nm.

Note: The λ_{max} for TNB is 412 nm, however most plate readers are equipped with a 405 nm filter.

Things to Note: (continued):

4. NADPH that has been stored for a prolonged time can be tested by demonstrating that the rate curve for the high calibrator is linear over a 5 minute interval.

5. Unused wells can be protected from contamination by used wells by sealing the used wells with 1 inch cellophane tape.

6. Samples containing GSH concentrations greater than the upper limit of the assay can usually be detected by observing very rapid color development. The rate curves of suspicious sample should be examined for linearity. Generally the reaction rate can be re-calculated over the linear portion of the rate curve and used to calculate the GSH concentration.

7. GSH is rapidly metabolized and will undergo mixed disulfide reactions; therefore, it is recommended that samples be processed as soon as possible to avoid the loss of GSH.

8. Please contact NWLSS for advice if the expected GSH concentration in the samplers is near or below the sensitivity of the standard method.

References:

1. Halliwell, B.; Gutteridge, J.M.C. Free Radicals in Biology and Medicine. Oxford University Press, New York; 1999.

2. Teitze, F. Enzymatic method for the quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues, *Anal. Biochem.***27:**502-522; 1969.

Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

Performance Details (continued):

Dynamic Range:

The linearity and sensitivity of the calibration curve defines the useful range of the assay. As shown in Figure 5, an unknown sample with a GSH concentration, following dilution in Assay Buffer, greater than the 20 µM will not be accurate and should be further diluted and re-assayed.

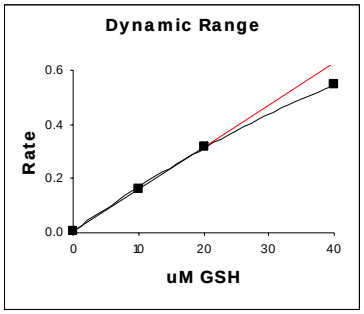

Figure 5.
The calibration curve (—) is compared to the plot of a series of GSH concentrations that exceed the range of the assay (■).

Stability

All unopened reagents are stable until the expiration date stated on the package label when stored at 2-8°C.

Reconstituted NADPH is usable for up to 1 week if stored at 2-8°C and up to 1 month at -20°C if stored sealed and uncontaminated in the original bottle.

Deproteinated samples in 5% MPA are stable up to 6 months at -20°C or indefinitely at -80°C.

Things to Note:

1. Endpoint assay is not recommended; kinetic is more precise and faster.

2. The reagents are provided with a reasonable overfill but use caution if using multi-channel pipetting troughs to minimize reagent volume loss.

3. Do not pre-combine the NADPH, DTNB and GR. GR will catalyze the reduction of DTNB by NADPH resulting in higher background and/or exhaustion of reagents.

Warnings, Limitations, Precautions:

NADPH

β-Nicotinamide adenine dinucleotide phosphate, reduced form (CAS 2646-71-1) is irritating to the eyes, respiratory system and skin. Target organs: nerves, liver. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing.

MPA

Metaphosphoric acid (CAS 37267-86-0) is corrosive and may cause burns. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear protective clothing, gloves and eye protection.

DTNB

5-5′-Dithiobis(2-nitrobenzoic acid) (CAS 69-78-3) is irritating to the eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing.

Interference

Thiol containing compounds, such as cysteine, β-mercaptoethanol or dithiothreitol may compete with GSH for DTNB. N-ethylmaleimide or other thiol alkylating agents are known to interfere with GR and also will react with GSH and should be avoided.

Storage Instructions:

Upon receipt, store the reagents at 2-8ºC. Do not use components beyond the expiration date printed on the label.
All reagents should be brought to room temperature (18-25°C) prior to use.

Assay Preparation

Plate Reader Setup Recommendations

Wavelength: 405 nm

Duration: 3 minutes

Interval: 15-20 seconds

Data Reduction: Linear regression

nwkgsh01rev010307

Reagent Preparation:

5% Metaphosphoric acid

1. Weigh 1 gram of MPA into a beaker or flask
2. Add 20 mL deionized water to the beaker or flask
3. Stir until dissolved
4. Store at 2-8°C until use

Note: 5% MPA solution should be prepared and used the same day.

NADPH

1. Add the entire contents of one NADPH Diluent bottle to a NADPH bottle.
2. Secure cap on vial containing the reconstituted NADPH and mix briefly by inverting the bottle.

Note: Once reconstituted, the NADPH is usable for up to 1 week if stored at 2-8ºC in the original container. The NADPH reagent can also be stored at -20°C for up to 6 weeks. However, it is recommended that the reagent be tested before committing samples.

Calibrators

Add 50 µL GSH Calibrator to 950 µL Assay Buffer = 20 µM GSH Equiv.
Add 25 µL GSH Calibrator to 975 µL Assay Buffer = 10 µM GSH Equiv.
Assay Buffer Only = 0 µM GSH equivalents.

Other Reagents

The DTNB, GR and Assay Buffer are supplied ready-to-use.

Sample Handling/Preparation:

The multi-disciplinary interest in measuring GSH has resulted in a myriad of sample types and experimental conditions and is beyond the scope of this product insert to describe sample processing in detail. However, general guidelines are provided below for representative sample types. If additional information is required, please contact NWLSS to discuss the particular sample under investigation.

Deproteination

Most samples will require removal of interfering proteins and metabolizing enzymes prior to assay. The acidic environment of the deproteinated sample also improves the stability of GSH.

1. Add 50 µL of sample to microcentrifuge tube.
2. Add 100 µL cold 5% MPA.
3. Vortex for 5 seconds (5 count).
4. Centrifuge at >1,000 x*g* for 5 minutes or equivalent.

Data Analysis: (continued)

Continuing with the example above…

- The unknown rate = 0.197 A_{405}/min
- The unknown *df* (1/3 deproteination and 1/21 buffer dilution) = 63x

Substituting the values into Equation 2, the GSH concentration for the unknown was found to be…

$$[GSH] = \frac{0.197\text{-}0.0091}{0.0146} \bullet 63 = 810.8\ \mu M$$

Performance Details:

Precision

The precision of the assay was estimated by measuring a set of controls having low and high GSH concentrations, in duplicate, two times each day for five consecutive days using the standard procedure.

Control	µM GSH	Intra-assay		Inter-assay		Total	
	Mean	s	%CV	s	%CV	s	%CV
Low	219	6	2.7	9	4.1	7	3.2
High	827	11	1.3	14	1.7	12	1.5

Sensitivity

The method detection limit or MLD is estimated using twice the standard error of the rate of the calibration curve or 0.5 µM GSH. Alternatively, the lower limit of detection (LLD) is defined as 3.29 standard deviations from zero or 0.1 µM.

Accuracy

Recovery: A 10 mM solution of GSH was diluted in Assay Buffer to 20 µM and measured against the GSSG calibrators using the standard method. The GSH recovery was 102% showing the equivalency of the GSSG calibrators in the assay.

Data Analysis: (continued)

The calibration curve, shown in Figure 4, is constructed by plotting the rate for each calibrator as a function of the GSH concentration.

The general equation for the GSH calibration curve is:

$$\text{Rate} = a[\text{GSH}] + b \qquad \textbf{Equation 1}$$

where *a* and *b* are the slope and intercept of the linear regression equation, respectively.

Linear regression analysis of the rates as a function of concentration from Table 1 yielded the following equation:

Rate = 0.01464[GSH] + 0.0091, r^2 = 1.000.

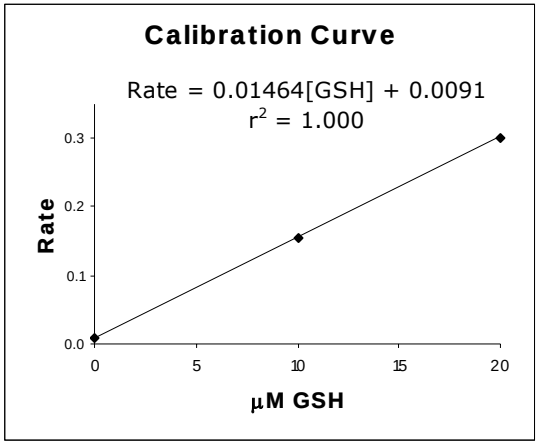

Figure 4.
Plot of the increase in the rate (ΔA_{405}/min) as a function of the concentration of GSH.

Rearranging Equation 1, the concentration of GSH in the unknown is determined given the rate...

$$[\text{GSH}] = \frac{\text{Rate} - b}{a} \bullet df \qquad \textbf{Equation 2}$$

where *df* is the dilution factor for the unknown.

Deproteination (continued):

5. Carefully remove supernatant.
6. Place supernatant on ice or store at -20°C if sample is to be assayed at a later date.
7. Just prior to assay, dilute sample at least 1/20 in Assay Buffer

Note: If GSH is to be normalized to hemoglobin or protein, these tests must be performed on non-deproteinated samples.

Whole Blood:

Collect samples using EDTA, heparin, citrate or ACD anticoagulant. Store at 2-8°C. DO NOT FREEZE. Deproteinate prior to assay.

Tissue

The GSH concentration in most tissues ranges from 1-10 mM; therefore, a 10% w/v homogenate is recommended. Contaminating blood contains significant concentrations of GSH and, if practical, should be removed by perfusion with an appropriate buffer, such as phosphate Buffered saline containing heparin. Clarify homogenates by centrifugation and store the supernatant on ice. Deproteinate as soon as possible to reduce the loss of GSH to various metabolic enzymes and mixed disulfide reactions. The GSH concentration can be normalized to the wet weight of the tissue sample or to the protein concentration of the homogenate.

Cultured Cells

Prepare a cell suspension of approximately 10^6 cells per mL in an appropriate buffer and homogenize or disrupt the cells by sonication. Clarify the homogenate by centrifugation and store the supernatant on ice. The GSH concentration can be normalized to the cell number or to the protein concentration of the homogenate. Deproteinate as soon as possible to reduce the loss of GSH to various metabolic enzymes and mixed disulfide reactions. It is recommended that a trial assay with a representative sample be tested to determine if the samples are within the dynamic range of the assay. If the test sample is below the sensitivity of the standard procedure, please contact NWLSS for assistance.

Plasma

The GSH concentration in plasma can be at the limits of sensitivity of the assay following standard deproteination and dilution. Therefore, it may be necessary to use the high sensitivity protocol as posted at www.nwlifescience.com/products/assaykit/gshhighsens.htm
.

Sample Handling/Preparation (continued):

Oxidized Glutathione

Oxidized GSH (GSSG) can be estimated using the NWLSS GSH Assay by first scavenging any free GSH in the sample by incubating the homogenate with of 4-vinylpyridine for 60 minutes at room temperature. The concentration of 4-vinylpyridine (4-VP, Sigma V-3877 or equivalent) in the homogenate should be approximately 10-fold greater than the expected GSH concentration. Because of measurable interference by 4-VP, the calibrators must be treated in the same manner as the samples. The GSSG in most samples is at or below the detection limit of the standard assay method however a modified protocol for testing GSSG is posted at http://www.nwlifescience.com/products/PDF/gsh01gssg.htm for our customers wishing to measure oxidized glutathione.

Assay Protocol:

Standard Procedure

1. Bring all reagents to room temperature.
2. Remove microplate from plastic bag.
3. Add 50 µL of calibrator, diluted sample, and diluted control to a designated well.
4. Add 50 µL of DTNB to each well.
5. Add 50 µL of GR to each well
6. Incubate microplate for 3-5 minutes at room temperature.
7. Add 50 µL of reconstituted NADPH to each well.
8. Begin recording the absorbance at 405 nm at 15-20 second intervals for 3 minutes.
9. Determine the concentration of the controls and samples. If using data reduction on the plate reader, skip steps a-d.
   a. Calculate the rate for each calibrator, control and sample from the slope of the linear regression of A_{405} as a function of time.
   b. Calculate the linear regression parameters to obtain the equation of the line.
   c. Calculate the concentrations of the controls and samples.
   d. Correct the control and sample for dilution and report results.

Data Analysis:

The following example shows the expected behavior and results of GSH determination using the standard method with the NWLSS™ Glutathione assay.

A 50 µL sample of whole blood sample was added to a microcentrifuge tube containing 100 µL cold 5% MPA. The microcentrifuge tube was then vortexed, centrifuged and the supernatant collected and placed on ice. Following the 25:500 dilution in Assay Buffer, the diluted sample was assayed using the standard method.

Data Analysis (continued):

The plot of the absorbance as a function of time for each calibrator and the unknown sample are shown in Figure 3. On inspection the curves are linear as expected.

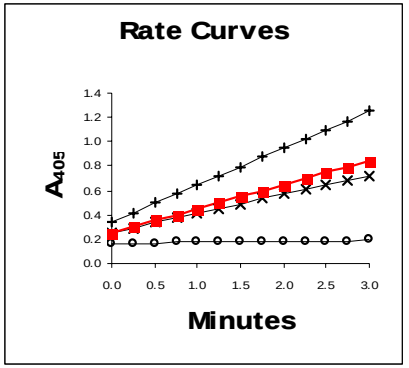

Figure 3.
Plot of the A_{405} as a function of time for calibrators 0(○), 10 µM (x), 20 µM (+) GSH and unknown (■).

Alternatively, the r^2 parameter can be used as a measure of linearity (Table 1). If curves appear to be non-linear or have an r^2 value less than 0.995, then that sample should be repeated following further dilution, see Dynamic Range, page 11.

For each curve in Figure 3, the reaction rate (rate = slope) for each curve was determined using linear regression analysis. The rate for each calibrator and the unknown sample are shown in Table 1.

Sample		Rate ΔA_{405}/min	r^2
Calibrator	0 µM	0.0088	0.996
Calibrator	10 µM	0.1560	1.000
Calibrator	20 µM	0.3016	1.000
Unknown		0.1972	1.000

Table 1: Reaction Rates