Introduction

Glutathione (GSH, γ-glutamylcysteinylglycine), the primary non-protein sulphydryl 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.

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 and is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation.¹

Intended Use

The NWK-GSH01™ 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 the 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.
General Specifications

Format: 2x96 wells
Number of tests: Triplicate = 58
Duplicate = 90
Specificity: Total Glutathione (GSH plus GSSG)
Sensitivity: LLD = 0.1 µM in the assay
LLD = 6.3 µM in the original sample

Kit Contents

NADPH β-Nicotinamide adenine dinucleotide phosphate, reduced; 2 vials dry powder
NADPH Diluent Buffer with stabilizer; 2 x 6 mL vials
DTNB 5-5’-Dithiobis(2-nitrobenzoic acid) in phosphate buffer with EDTA; 1x11 mL
GR Glutathione reductase in Assay Buffer with protein stabilizer; 1x11 mL
Calibrators Glutathione disulfide in Assay Buffer at 0, 10, 20 µM GSH equivalents; 3x1 mL
Assay Buffer Phosphate buffer with EDTA, pH 7.6; 1x125 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 $\lambda_{\text{max}}$ for TNB is 412 nm, however most plate readers are equipped with a 405 nm filter.

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 2-8°C. 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

Reagent Preparation

5% Metaphosphoric acid

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

Note: The 5% MPA solution should be prepared and used on 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 2-8°C in the original container. The reagent can also be stored at -20°C for up to 6 weeks. However, it is recommended that the reagent be tested before committing valuable samples – see Notes.
Other Reagents

The DTNB, GR, calibrators 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. Please contact NWLSS to discuss the particular sample under investigation.

Deproteination

Most samples will require removal of interfering proteins and metabolizing enzymes. In addition, the acidic environment of the deproteinated sample improves the stability of GSH. The recommended procedure is listed below.

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.
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 as the anticoagulant. Store at 2-8°C. DO NOT FREEZE.

Tissue

The GSH concentration in most tissues ranges from 1-10 mM; therefore, a 10% w/v homogenate is recommended. Contaminating blood contains high concentrations of GSH and, if practical, should be removed by perfusion with an appropriate buffer, such as phosphate buffered saline containing heparin. Clarify the homogenate by centrifugation and store the supernatant on ice. The sample and buffers should be kept cold and the homogenate should be deproteinized 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. The sample and buffers should be kept cold and the homogenates should be deproteinized 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 is at the limits of sensitivity following standard deproteination and dilution. Therefore, plasma samples require a modification of the standard assay procedure. Please contact NWLSS for assistance.

Oxidized Glutathione

Oxidized GSH (GSSG) can be estimated using the NWK-GSH01™ 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 assay. Please contact NWLSS for assistance.

Protocol

Standard Procedure

1. Remove microplate from plastic bag.
2. Bring all reagents to room temperature.
3. Add 50 µL of calibrator, diluted sample, and diluted control to a designated well.
4. Add 50 µL of DTNB to wells containing calibrator, control or sample.
5. Add 50 µL of GR to wells containing calibrator, control or sample.
6. Incubate microplate for 3-5 minutes at room temperature.
7. Add 50 µL of reconstituted NADPH to wells containing calibrator, control or sample.
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.
   1. Calculate the rate for each calibrator, control and sample from the linear regression of A405 as a function of time.
   2. Calculate the linear regression parameters to obtain the equation of the line.
   3. Calculate the concentrations of the controls and samples.
   4. 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 NWK-GSH01™ 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.

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.

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

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.
Figure 3. Plot of the $A_{405}$ as a function of time for calibrators (•) and unknown (○).

Table 1. Reaction Rates

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta A_{405}/\text{min}$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator 0 µM</td>
<td>0.0088</td>
<td>0.996</td>
</tr>
<tr>
<td>Calibrator 10 µM</td>
<td>0.1560</td>
<td>1.000</td>
</tr>
<tr>
<td>Calibrator 20 µM</td>
<td>0.3016</td>
<td>1.000</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.1972</td>
<td>1.000</td>
</tr>
</tbody>
</table>

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

Figure 4. Calibration Curve.

The general equation for the GSH calibration curve is:

$$\text{Rate} = a[GSH] + b \quad \text{Equation 1.}$$
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:

\[
\text{Rate} = 0.01464[GSH] + 0.0091, \quad r^2 = 1.000.
\]

Figure 4. Plot of the increase in the rate as a function of the concentration of GSH.

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

\[
[GSH] = \frac{\text{Rate} - b}{a} \cdot df
\]

Equation 2

where \( df \) is the dilution factor for the unknown.

Continuing with the example above...

- The unknown rate = 0.197 A\textsubscript{405}/mL
- The unknown dilution factor (1/3 deproteination and 1/21 buffer dilution) = 63x

Substituting into Equation 2, the GSG concentration for the unknown was found to be...

\[
[GSH] = \frac{0.197 - 0.0091}{0.0146} \cdot 63 = 810.8 \text{ µM}
\]

### 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.

<table>
<thead>
<tr>
<th>Control</th>
<th>µM GSH Intra-assay Inter-assay Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Mean 6 s 2.7%CV s 9 %CV 7.1%CV s 7.3%CV s 3.2%CV</td>
</tr>
<tr>
<td>High</td>
<td>Mean 11 %CV s 1.3%CV s 14 %CV 1.7%CV s 1.5%CV</td>
</tr>
</tbody>
</table>

#### Sensitivity

The sensitivity is estimated using the 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% which shows the equivalency of the GSSG calibrators in the assay.
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.

![Dynamic Range](image)

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 Notes**

1. Endpoint assay is not recommended; kinetic is more precise and faster
2. The reagents are provided with a reasonable overfill but use caution when 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.
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. Supernatants containing GSH in 5% MPA can be stored at less than -20°C for at least 6 months. Upon thawing, it is important to thoroughly mix the sample to ensure accurate sampling.
9. Please contact NWLSS for advice if the expected GSH concentration in the samplers is
near or below the sensitivity of the standard method.

References


Procedure Checklist

- Process the sample and place on ice
- Prepare 5% MPA and place on ice
- Bring GSH reagents to ambient temperature
- Setup microplate reader
  - 405 nm
  - Kinetic mode
  - 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.