

## > Total Glutathione Detection Kit

Catalog # 900-160

Sufficient Reagents for 384 tests with 4 x 96-well plates  
For use with mammalian cell and tissue extracts, whole blood,  
plasma, urine, and saliva

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All reagents should be stored at 4°C.



Check our website for additional protocols, technical notes and FAQs.



For proper performance, use the insert provided with each individual kit received.

**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

## Introduction

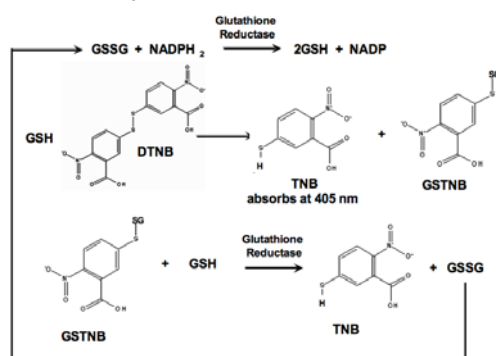
Assay Designs' Total Glutathione Activity Kit is a complete kit for the measurement of total, reduced, and oxidized glutathione concentration in mammalian cell and tissue extracts, whole blood, plasma, saliva, and urine.

Increased oxidative damage of proteins, lipids, and DNA by free radicals is one of the pathogenic mechanisms of diseases such as cancer, atherosclerosis, inflammation, and neurodegenerative disorders<sup>1,2</sup>. Glutathione, a major intracellular non-protein thiol, protects against free radical damage by providing reducing equivalents for several key antioxidant enzymes. In addition Glutathione acts as a scavenger of hydroxyl radicals and nascent oxygen. Markedly reduced Glutathione levels are associated with a reduced rate of illness, higher levels of self-rated health, lower cholesterol, lower body mass index, and lower blood pressure among the elderly<sup>3</sup>. Glutathione provides a primary defense system for the removal of oxidants in the brain. Studies reveal a correlation between low Glutathione levels and damage to neurons that manufacture dopamine, suggesting a link to Parkinson's disease<sup>4</sup>. The concentration of Glutathione ranges from 10 $\mu$ m to 1mM in cells and is in the micromolar range in plasma.

The Total Glutathione Assay utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). As shown in Figure 1, the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that absorbs at 405 or 414 nm. The rate of TNB production is directly proportional to the concentration of glutathione in the sample. The measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of glutathione in the sample.

## Principle

1. Samples and diluted standards are added to wells of a 96-well plate.
2. Reaction Mix is added to the wells to initiate the reaction.
3. The plate is transferred to a plate reader and absorbance readings are taken at 405 nm or 414 nm every minute for 10 minutes.



## Materials Supplied

- 1. Clear Microtiter Plate**  
Eight Plates of 96 Wells, Catalog No. 80-1639  
Clear uncoated solid plates.
- 2. 25X Assay Buffer**  
12 mL, Catalog No. 80-1669
- 3. Reaction Mix**  
8 bottles, Catalog No. 80-1667  
One vial is sufficient for 53 wells of a 96 well plate.
- 4. GSSG (4  $\mu$ M)**  
2.5 mL, Catalog No. 80-1666
- 5. Glutathione Reductase**  
80  $\mu$ L, Catalog No. 80-1665
- 6. Total Glutathione Assay Layout**  
(See Figure 2)

## Storage

All components of this kit are stable at 4°C. All kit components are stable at their recommended storage temperatures until the kit expiration date.

## Materials Needed but Not Supplied

1. PBS
2. Distilled water
3. Metaphosphoric Acid (Aldrich Catalog No. 23,927-5)
4. 4-Vinylpyridine (Aldrich Catalog No. V3204-5ML)
5. Reagent alcohol
6. Microtubes, 0.5 and 1.5 mL
7. 15 mL conical tubes (adherent and suspension cell preparation)
8. 50 mL conical tubes (tissue preparation)
9. Precision pipettes for volumes between 5-1000  $\mu$ L
10. Multichannel pipettor for volumes between 1 - 50  $\mu$ L and 50  $\mu$ L – 200  $\mu$ L
11. Microplate reader capable of reading at 405 or 414 nm and taking readings every minute for ten minutes and exporting data to an Excel spreadsheet
12. Centrifuge and microfuge for processing samples
13. Sonicator or Homogenizer



Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.



The physical, chemical, and toxicological properties of the chemicals and reagents contained in this kit may not yet have been fully investigated. Therefore, we recommend the use of gloves, lab coats, and eye protection while using any of these chemical reagents.



All solutions must be prepared just before use.



Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.



Warning: Prepare and use 2M 4-Vinylpyridine solution in a chemical fume hood.

## Reagent Preparation

### 1. 1X Assay Buffer

Allow the assay buffer to come to room temperature for a minimum of 30 minutes. Dilute the 25X Assay Buffer to 1X (1:25) with distilled water. The 1X Assay Buffer is used to prepare dilutions of Total Glutathione Standard Curve and to dilute each experimental sample.

### 2. Reaction Mix

Reconstitute one or more bottles of Reaction Mix with 8 mL of distilled water. Periodically swirl the bottle gently over a 15 minute period to dissolve contents. **Immediately before use in the assay, vortex the vial of Glutathione Reductase and add 10  $\mu$ L of the Glutathione Reductase to the bottle of Reaction Mix.** Each bottle of Reaction Mix is sufficient for 53 wells in a 96-well plate, or little more than half a plate. Pool the reconstituted Reaction Mix together into one tube if more than one bottle is used.

### 3. 5% (w/v) Metaphosphoric acid

Prepare 5% (w/v) Metaphosphoric acid (Aldrich Catalog No. 23,927-5, not provided) in distilled water.

### 4. 2M 4-Vinylpyridine

Prepare 2M 4-vinylpyridine solution (Aldrich Catalog No. V3204-5ML, not provided) by mixing 108  $\mu$ L 4-vinylpyridine with 392  $\mu$ L ethanol (solution should be prepared and subsequently used only in a chemical fume hood). Use immediately and discard any unused portion. Note: It is recommended that you use 4-vinylpyridine within 1 month of purchase and store at  $-20^{\circ}\text{C}$ . This reagent blocks free thiols present in the reaction, thus eliminating any contribution to the cycling reaction caused by GSH.

### 5. Experimental Samples

After preparing the samples as outlined in the Sample Handling section that follows, dilute each sample 1:10 with 1X Assay Buffer. Some samples such as whole blood, liver, or red blood cells, may need to be diluted 1:20, 1:40, or more. It may be necessary to make serial dilutions of your extracts to obtain a satisfactory change in absorbance readings with time.

## Sample Handling

All samples are treated with 5% (w/v) Metaphosphoric acid to remove proteins which interfere with the assay.

### Cell Lysate Preparation

1. Detach **adherent cells** by gentle trypsinization. Count the cells and centrifuge at 300 x g for 10 minutes at 4°C. Wash the cells once with cold 1X PBS. Centrifuge **suspension cells** at 300x g for 10 minutes at 4°C. Discard the supernatant. Wash the cells once with cold 1X PBS.
2. Suspend the pellet with 500 µL of cold 5% (w/v) Metaphosphoric acid per 2-5 x 10<sup>6</sup> cells. Mix thoroughly by repeated pipetting. Homogenize or sonicate the cell suspension and store on ice for 5 minutes.
3. Transfer the suspension to a 1.5 mL tube and centrifuge at 12,000-14,000 x g for 5 minutes at 4°C. Place the supernatant into a clean 1.5 mL tube. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

### Tissue Lysate Preparation

1. Remove tissue. Repeatedly wash the tissue with cold isotonic saline (150 mM) or 1x PBS with 0.16 mg/mL heparin to prevent coagulation.
2. Blot tissue on filter paper and weigh.
3. Add ice-cold 5% (w/v) Metaphosphoric acid (20 mL/g tissue) and homogenize using a cold glass or teflon pestle.
4. Centrifuge the homogenate at 12,000-14,000 x g for 10-15 minutes at 4°C.
5. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

### Erythrocyte Lysate Preparation

1. Collect blood in Vacutainers containing heparin or sodium citrate as anticoagulant. Centrifuge at 3,000 x g for 10-15 minutes at 4°C.
2. Discard as much of the plasma supernatant as possible. Remove the white buffy coat (leukocytes) on the surface of the erythrocytes.
3. Resuspend the erythrocyte pellet in four volumes of ice-cold 5% (w/v) Metaphosphoric acid. Mix thoroughly 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 clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

### **Whole Blood Lysate Preparation**

1. Collect blood in tubes containing heparin or sodium citrate as anticoagulant.
2. Add four volumes of ice-cold 5% (w/v) Metaphosphoric acid. Mix thoroughly and store on ice for 15 minutes.
3. Centrifuge at 12,000-14,000 x g for 10-15 minutes at 4°C.
4. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

### **Urine, Plasma, and Saliva Preparation**

1. Collect urine, plasma, or saliva and immediately add four volumes of ice-cold 5% (w/v) Metaphosphoric acid. Mix thoroughly and store on ice for 15 minutes.
2. Centrifuge at 12,000-14,000 x g for 10-15 minutes at 4°C.
3. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

## Assay Procedure

### A. Total Glutathione Assay

1. Set up the Glutathione standard curve
  - a. Add 50  $\mu\text{L}$  of 1X Assay Buffer to all the wells in rows A through E, columns 1, 2, and 3 of the microtiter plate (see Figure 2).
  - b. Add 50  $\mu\text{L}$  of the 4  $\mu\text{M}$  GSSG to wells A1, A2, and A3 with a multichannel pipetor. Mix well by pipeting the solution up and down at least ten times.
  - c. Transfer 50  $\mu\text{L}$  from wells A1, A2, and A3 to wells B1, B2, and B3, respectively. Mix well at least 10 times and transfer 50  $\mu\text{L}$  from row B to row C. Continue in this fashion to row D. Mix and discard the last 50  $\mu\text{L}$  from row D. Wells E1, E2, and E3 are set aside as Blank wells. The GSSG content in rows A, B, C, and D, is 100 pmoles/well, 50 pmoles/well, 25 pmoles/well, and 12.5 pmoles/well, respectively.
2. Add 50  $\mu\text{L}$  of your diluted experimental samples to the wells in columns 4 to 12. **Note:** It may be necessary to make serial dilutions of your extracts to obtain a satisfactory change in absorbance readings with time.
3. Prior to the next step, set up the parameters of your plate reader to measure absorbance at 405 nm or 414 nm and to read the required wells. Include a 10 second orbital shake prior to the initial read.
4. Using a multichannel pipetor, add 150  $\mu\text{L}$  of freshly-prepared Reaction Mix to each well.
5. Immediately record the absorbance in the wells at 405 nm or 414 nm using a plate reader at 1 minute intervals over a 10 minute period. **Note:** If you intend to use all the wells on one plate in the assay, it may be necessary to record the absorbance at 2 minute intervals.

### B. Oxidized Glutathione Assay

1. Add 1  $\mu\text{L}$  of 2M 4-vinylpyridine per 50  $\mu\text{L}$  of sample and 4  $\mu\text{M}$  GSSG. Incubate for one hour at room temperature (cell lysates should be diluted at least 1:10 prior to 4-vinylpyridine treatment).
2. Serially dilute the 4-vinylpyridine-treated GSSG standard as described above in the total glutathione assay protocol (Step A.1).
3. Serially dilute your 4-vinylpyridine-treated experimental samples as described above in the total glutathione assay protocol (Step A.2).
4. Follow steps 3, 4, and 5 as described above for the total glutathione assay.



All standards, controls, and samples should be run in triplicate.



Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.



Pipet the reagents to the sides of the wells to avoid possible contamination.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	GSSG 100 pmole	GSSG 100 pmole	GSSG 100 pmole	Sample 2 1:10	Sample 2 1:10	Sample 2 1:10	Sample 4 1:40	Sample 4 1:40	Sample 4 1:40	Sample 7 1:20	Sample 7 1:20	Sample 7 1:20
<b>B</b>	GSSG 50 pmole	GSSG 50 pmole	GSSG 50 pmole	Sample 2 1:20	Sample 2 1:20	Sample 2 1:20	Sample 5 1:10	Sample 5 1:10	Sample 5 1:10	Sample 7 1:40	Sample 7 1:40	Sample 7 1:40
<b>C</b>	GSSG 25 pmole	GSSG 25 pmole	GSSG 25 pmole	Sample 2 1:40	Sample 2 1:40	Sample 2 1:40	Sample 5 1:20	Sample 5 1:20	Sample 5 1:20	Sample 8 1:10	Sample 8 1:10	Sample 8 1:10
<b>D</b>	GSSG 12.5 pmole	GSSG 12.5 pmole	GSSG 12.5 pmole	Sample 3 1:10	Sample 3 1:10	Sample 3 1:10	Sample 5 1:40	Sample 5 1:40	Sample 5 1:40	Sample 8 1:20	Sample 8 1:20	Sample 5 1:20
<b>E</b>	Back- ground	Back- ground	Back- ground	Sample 3 1:20	Sample 3 1:20	Sample 3 1:20	Sample 6 1:10	Sample 6 1:10	Sample 6 1:10	Sample 8 1:40	Sample 8 1:40	Sample 8 1:40
<b>F</b>	Sample 1 1:10	Sample 1 1:10	Sample 1 1:10	Sample 3 1:40	Sample 3 1:40	Sample 3 1:40	Sample 6 1:20	Sample 6 1:20	Sample 6 1:20	Sample 9 1:10	Sample 9 1:10	Sample 9 1:10
<b>G</b>	Sample 1 1:20	Sample 1 1:20	Sample 1 1:20	Sample 4 1:10	Sample 4 1:10	Sample 4 1:10	Sample 6 1:40	Sample 6 1:40	Sample 6 1:40	Sample 9 1:20	Sample 9 1:20	Sample 9 1:20
<b>H</b>	Sample 1 1:40	Sample 1 1:40	Sample 1 1:40	Sample 4 1:20	Sample 4 1:20	Sample 4 1:20	Sample 7 1:10	Sample 7 1:10	Sample 7 1:10	Sample 9 1:40	Sample 9 1:40	Sample 9 1:40

Figure 2. Suggested 96-well plate format for GSSG Standards and samples.

## Calculation of Results

### **A. Determination of Total Glutathione Concentration**

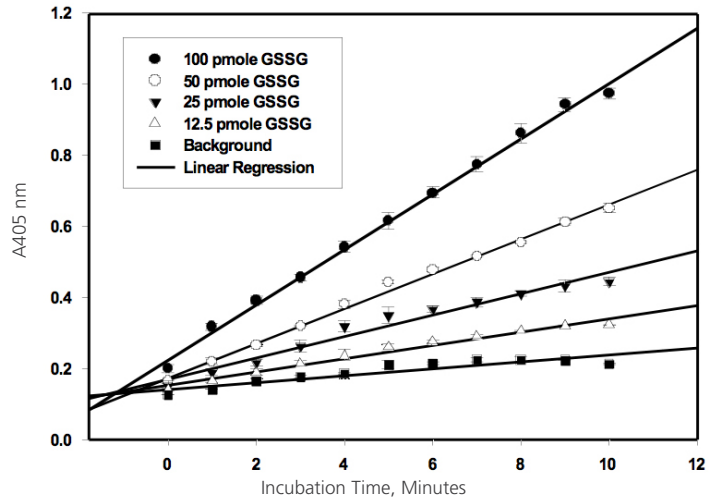
1. Take the average of the triplicate absorbance readings for each standard, sample, and Blank at each time point.
2. Plot the average of each standard, sample, and background absorbance (A405 nm) versus incubation time and determine the slope from the linear portion of each curve (Figure 3).
3. Subtract the background slope from the slopes of the standards and the experimental samples.
4. Plot the net slopes of the GSSG standards versus pmoles of Glutathione (Figure 4).
5. Compare the net slopes of the experimental samples with those of the standard curve from Figure 4 to determine the pmoles of GSSG (equivalent to total glutathione) for each experimental sample.

### **B. Determination of Oxidized Glutathione Concentration**

1. Follow the procedure described above for generating the Standard GSSG curves (Figures 3 and 4) for the 4-vinylpyridine treated standards.
2. Compare the net slopes of the 4-vinylpyridine-treated experimental samples with those of the 4-vinylpyridine-treated standard curve from Figure 4 to determine the pmoles of oxidized Glutathione for each experimental sample.
3. Subtract the pmole of oxidized glutathione in your sample from the pmole of total glutathione to obtain the pmole of reduced glutathione in your sample.

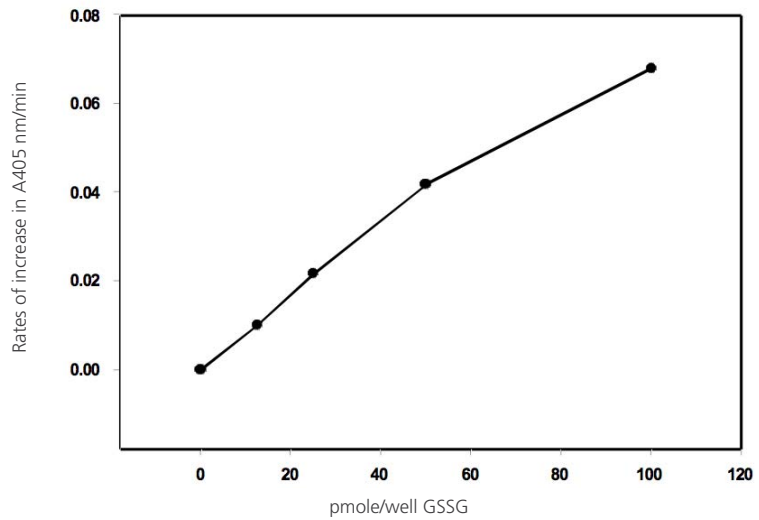
$$\text{Reduced GSH} = \text{Total glutathione} - \text{Oxidized GSSG}$$

**Change in Absorbance at 405 nm with Time as a Function of pmole/well of GSSG**



**Figure 3.** Plot of absorbance at 405 nm versus incubation time as a function of pmoles of GSSG/well.

**Graph of Rate of Increase in Absorbance at 405 nm versus pmole/well GSSG**



**Figure 4.** Rate of increase in the absorbance at 405 nm as a function of pmole/well GSSG.

## Trouble Shooting

Problem	Cause	Solution
Erratic within sample values	Inconsistent pipetting technique	Reproducible pipetting is absolutely required for consistent results
	Reaction Mix incompletely solubilized	Periodically vortex the Reaction Mix over a 15 minute period.
No color development in standards and samples	Failure to add Glutathione Reductase to Reaction Mix	Vortex Glutathione Reductase briefly and add 10 $\mu$ l to each bottle of reconstituted Reaction Mix.
	NADPH is oxidized	Contact Assay Designs
No color development in the samples but standards give color	Concentration of glutathione in the samples is below the sensitivity of the assay	Extend incubation time to 30 minutes.
Sample absorbance values higher than those of standard curve	Glutathione levels in the sample very high or other thiols are present	Further dilute your samples with 1X assay buffer and reassay

## References

1. Onyango IG. and Khan SM. Oxidative stress, mitochondrial dysfunction, and stress signaling in Alzheimer's disease. *Curr Alzheimer Res.* 2006 3:339-349.
2. Medina S., et al. 2003. Antioxidants inhibit the human cortical neuron apoptosis induced by hydrogen peroxide, tumor necrosis factor alpha, dopamine and beta-amyloid peptide 1-42. *Free Radic Res.* 36:1179-84.
3. Julius M., et al. 1994. Glutathione and morbidity in a community-based sample of elderly., *J Clin epidemiol.* 47: 1021-1026.
4. Mytilineou C, et al. 2002. Glutathione depletion and oxidative stress. *Parkinsonism Relat Disord.* 8:385-387.



MSDS (Material Safety Data Sheet) available online

## Limited Warranty

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

Assay Designs, Inc. tel: 734.668.6113 or 800.833.8651  
5777 Hines Drive fax: 734.668.2793  
Ann Arbor, Michigan info@assaydesigns.com  
USA www.assaydesigns.com

technical support: technical@assaydesigns.com



## Kits for Charity™

Assay Designs has always been an active contributor to a number of local, national and international charities. We have broadened our charitable contributions by implementing a program called Kits for Charity™.

Each quarter, Assay Designs will feature a different non-religious and non-political charitable organization on our website. For each kit sold during this time period, we will make a monetary contribution to the featured charity. Please check our website for the current quarter's charity to see what organization your purchases are helping to support.

If you have any suggestions for future Kits for Charity™ recipients, please contact us at 800.833.8651 or 734.668.6113.

## Contact Us

For more details concerning the information within this kit insert, or to order any of the Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

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734.668.6113 or 800.833.8651  
www.assaydesigns.com