Product Manual

Glutathione (GSSG/GSH), detection kit

Catalog # ADI-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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INTRODUCTION

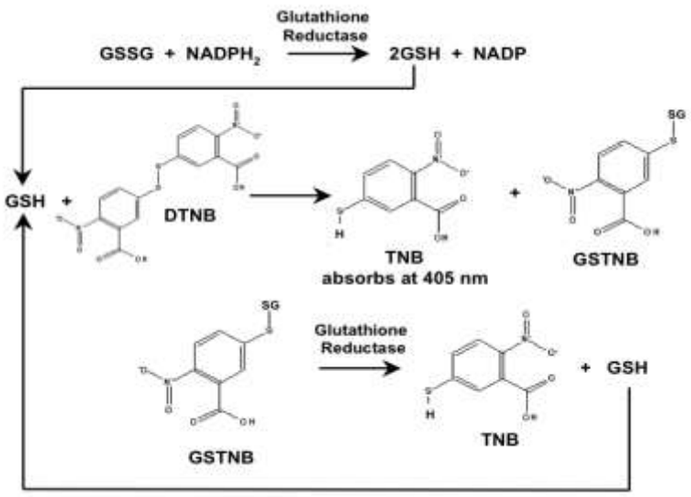
The Glutathione (GSSG/GSH), detection kit is a complete kit for the measurement of total, reduced, and oxidized glutathione concentration in mammalian cell and tissue extracts, whole blood, plasma, saliva, and urine.

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[1,2]. 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 scaven-ger of hydroxyl radicals and nascent oxygen. Elevated Glutathione levels are asso-ciated with a reduced rate of illness, higher levels of self-rated health, lower cho-lesterol, lower body mass index, and lower blood pressure among the elderly[3]. 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[4]. The concentration of Glutathione ranges from 10 µm to 1 mM in cells and is in the mi-cromolar range in plasma.

The Glutathione Assay utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione Reductase reduces oxidized glu-tathione (GSSG) to reduced glutathione (GSH). As shown in Figure 1, the sulfhy-dryl 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 ab-sorbs 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 PROVIDED

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

1. **Clear Microtiter Plate Pack, 2 Each, Catalog No. 80-2556**

   Two packs of four plates of 96 wells. Clear uncoated solid plates.

2. **25X Assay Buffer, 12 mL, Catalog No. 80-1669**

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.

3. **Reaction Mix, 8 bottles, Catalog No. 80-1667**

   One vial is sufficient for 53 wells of a 96 well plate.

4. **GSSG, 2.5 mL (4 µM), Catalog No. 80-1666**
5. **Glutathione Reductase, 80 µL, Catalog No. 80-1668**
6. **Total Glutathione Assay Layout**

   See Figure 2.

STORAGE

All reagents should be stored at 4°C.

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

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

OTHER MATERIALS NEEDED

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

1. PBS
2. Distilled water
3. Meta-Phosphoric acid (Sigma-Aldrich Catalog No. 239275)
4. 4-Vinylpyridine (Sigma-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 µL
10. Multichannel pipettor for volumes between 1 - 50 µL and 50 µL - 200 µL

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

REAGENT PREPARATION

1. **1X Assay Buffer**


All solutions must be prepared just before use.

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**


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

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 µL 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) meta-Phosphoric acid**

Prepare 5% (w/v) meta-Phosphoric acid (Sigma Aldrich Catalog No. 239275, not provided) in distilled water.

4. **2M 4-Vinylpyridine**


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

Prepare 2M 4-vinylpyridine solution (Sigma Aldrich Catalog No. V3204-5ML, not provided) by thawing at room temperature, then mixing 108 µL 4-vinylpyridine with 392 µL 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°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) meta-Phosphoric 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 4ºC. Discard the supernatant. Wash the cells once with cold 1X PBS.
2. Suspend the pellet with 500 µL of cold 5% (w/v) meta-Phosphoric acid per 2-5 x 10^6 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. To thaw, leave samples at room temperature until thawed.

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) meta-Phosphoric 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. To thaw, leave samples at room temperature until thawed.

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) Meta-Phosphoric. 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. To thaw, leave samples at room temperature until thawed.

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) meta-Phosphoric 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. To thaw, leave samples at room temperature until thawed.

Urine, Plasma, and Saliva Preparation

1. Collect urine, plasma, or saliva and immediately add four volumes of ice-cold 5% (w/v) meta-Phosphoric 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. To thaw, leave samples at room temperature until thawed.

PROCEDURAL NOTES

Total Glutathione Assay

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

1. Set up the Glutathione standard curve:

   a) Add 50 µL 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 µL of the 4 µM GSSG to wells A1, A2, and A3 with a multichannel pipettor. Mix well by pipetting the solution up and down at least ten times.

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

   c) Transfer 50 µL from wells A1, A2, and A3 to wells B1, B2, and B3, respectively. Mix well at least 10 times and transfer 50 µL from row B to row C. Continue in this fashion to row D. Mix and discard the last 50 µL 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.

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

2. Add 50 µL 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 pipettor, add 150 µL 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.

Oxidized Glutathione Assay

1. Add 1 µL of 2 M 4-vinylpyridine per 50 µL of sample and 4 µM 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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	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 1:2 0
B	GSSG 50 pmole	GSSG 50 pmole	GSSG 50 pmole	Sample 2 1:20	Sample 2 1:20	Sample 2 1:2 0	Sample 5 1:10	Sample 5 1:10	Sample 5 1:10	Sample 7 1:40	Sample 7 1:40	Sample 7 1:40
C	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 1:2 0	Sample 8 1:10	Sample 8 1:10	Sample 8 1:10
D	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 1:2 0
E	Back - ground	Back - ground	Back - ground	Sample 3 1:20	Sample 3 1:20	Sample 3 1:2 0	Sample 6 1:10	Sample 6 1:10	Sample 6 1:10	Sample 8 1:40	Sample 8 1:40	Sample 8 1:40
F	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 1:2 0	Sample 9 1:10	Sample 9 1:10	Sample 9 1:10
G	Sample 1 1:20	Sample 1 1:20	Sample 1 1:2 0	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 1:2 0
H	Sample 1 1:40	Sample 1 1:40	Sample 1 1:40	Sample 4 1:20	Sample 4 1:20	Sample 4 1:2 0	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

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.

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.

Reduced GSH = Total glutathione - Oxidized GSSG

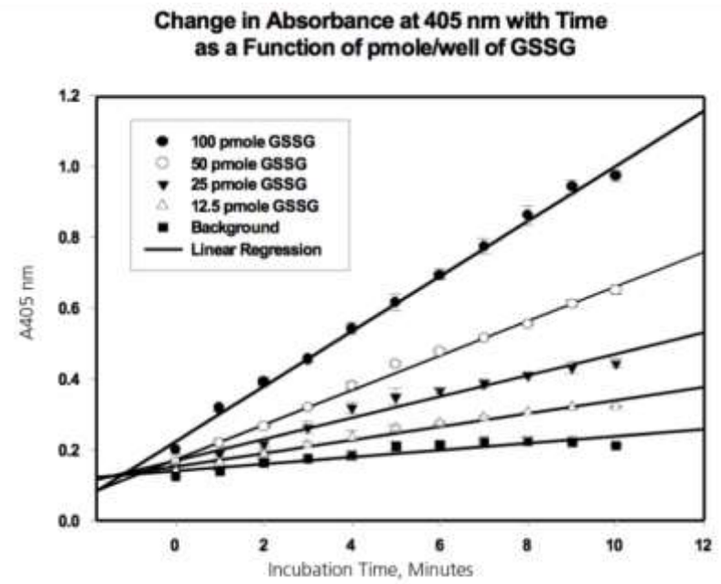


Figure 3. Plot of absorbance at 405 nm versus incubation time as a function of pmoles of GSSG/well.

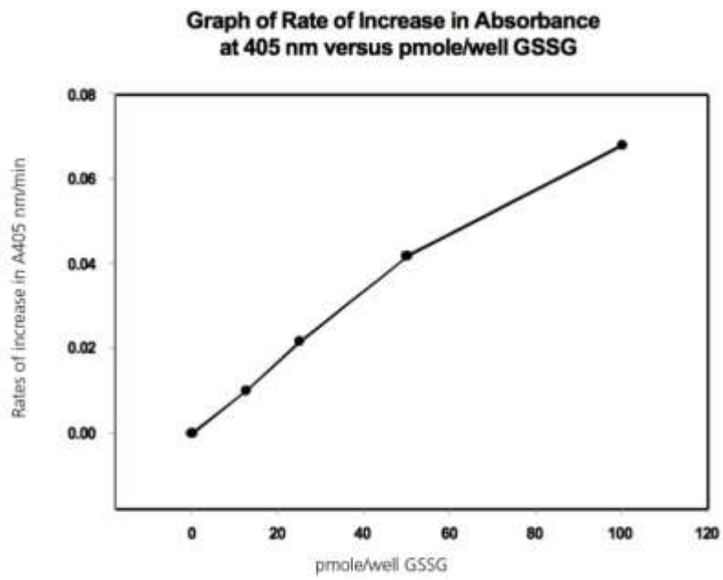


Figure 4. Rate of increase in the absorbance at 405 nm as a function of pmole/well GSSG.

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 µl to each bottle of reconstituted Reaction Mix
	NADPH is oxidized	Contact Technical Support
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

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