ELISA

HT Glutathione Assay Kit

Catalog Number: 7511-100-K

Colorimetric assay for total, reduced, and oxidized glutathione.

Sufficient reagents for 384 tests.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Increased oxidative damage by free radicals to proteins, lipids, and DNA is one of the pathogenic mechanisms of diseases such as cancer, atherosclerosis, inflammation, and neurodegenerative disorders (1, 2). Glutathione, the major intracellular non-protein thiol, is an important protector against free radical damage by both providing reducing agents for several key antioxidant enzymes as well as by scavenging hydroxyl radicals and nascent oxygen within a cell. Highly reduced glutathione levels are associated with fewer incidents of illness, higher levels of self-rated health, lower cholesterol, lower body mass index, and lower blood pressure in 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 1-10 mM in cells and is in the micromolar range in plasma.

The HT Glutathione Assay Kit is suitable for the quantification of glutathione in mammalian cells, tissue, blood, plasma, and other bodily fluids. It contains sufficient reagents to assay 384 data points or to determine glutathione levels in:

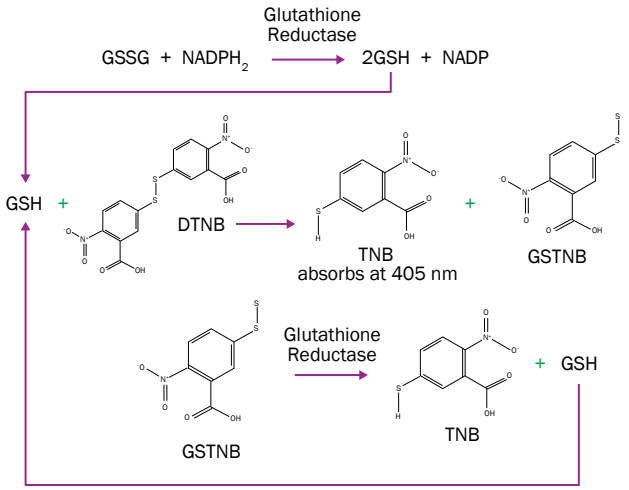
A) 123 samples, each performed in triplicate, plus one Glutathione Standard Curve.

B) 108 samples, each performed in triplicate, plus four Glutathione Standard Curves.

C) 88 samples, each performed in triplicate, plus eight Glutathione Standard Curves.

PRINCIPLE OF THE ASSAY

The HT Glutathione Assay Kit utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). The sulfhydryl group of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that absorbs at 405 nm or 414 nm. This mixed disulfide (GSTNB), is reduced by Glutathione Reductase to recycle the glutathione and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is directly proportional to the concentration of glutathione in the sample. The measurement of the absorbance of TNB at 405 nm or 414 nm provides an accurate estimation of glutathione in the sample.



LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTION

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

PART	PART #	AMOUNT PROVIDED	STORAGE OF UNOPENED MATERIAL
Glutathione Reductase	7511-100-01	80 µL	Store 2-8 °C.
25X Assay Buffer	7511-100-02	12 mL	
Reaction Mix	7511-100-04	8 bottles	
96 Well Plates	7511-100-05	8 plates	
4 µM GSSG	7511-100-06	2.5 mL	

OTHER SUPPLIES REQUIRED

Reagents

- Distilled water
- Cold 5% (w/b) Metaphosphoric Acid
- 2M 4-Vinylpyridine
- Reagent Ethanol
- 1X Phosphate Buffered Saline (PBS)

Equipment

- Microplate reader capable of measuring absorbance at 405 or 414 nm.
- Multi-channel pipette
- Pipettes and pipette tips
- Centrifuge (for cell lysis)
- Microcentrifuge tubes

REAGENT PREPARATION

1X Assay Buffer - Prior to each experiment, prepare the necessary amount of 1X Assay Buffer by diluting the 25X Assay Buffer (1:25) with distilled water.

Reaction Mix - Reconstitute the necessary amount (one or more bottles) of Reaction Mix with 8.0 mL of distilled water per bottle. Vortex each bottle until the contents completely dissolve (~10 seconds).

Glutathione Reductase Reaction Mix - Immediately before use in the assay, vortex the vial of Glutathione Reductase and add 10 µL to one 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.

5% (w/v) Metaphosphoric Acid - Prepare 5% (w/v) Metaphosphoric Acid in distilled water. Store on ice until ready to use assay.

2M 4-Vinylpyridine - This reagent blocks free thiols present in the reaction, thus eliminating any contribution to the cycling reaction caused by GSH. Prepare 2M 4-Vinylpyridine solution by 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 4-Vinylpyridine is used within 1 month of purchase and stored at ≤ -20 °C.*

SAMPLE PREPARATION & STORAGE

All samples are treated with 5% (w/v) Metaphosphoric Acid to remove proteins that 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 2-8 ˚C. Wash the cells once with cold 1X PBS.
2. Suspend the pellet with 500 µL of ice cold 5% (w/v) Metaphosphoric 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 microcentrifuge tube and centrifuge at 12,000-14,000 x g for 5 minutes at 2-8 ˚C. Place the supernatant into a clean 1.5 mL microcentrifuge tube. Store on ice if you intend to immediately assay for glutathione. Freeze at ≤ -70 ˚C for future use.

SAMPLE COLLECTION & STORAGE *CONTINUED*

Tissue Lysate Preparation

1. Remove as much blood as possible by perfusing the tissue with cold isotonic saline (150 mM NaCl) or 1X PBS containing heparin (0.16 mg/mL) to prevent coagulation.
2. Wash the tissue with cold isotonic saline (150 mM NaCl) or 1X PBS. 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 2-8 ˚C.
5. Collect the clarified supernatant. Store on ice if you intend to immediately assay for glutathione. Freeze at ≤ -70 ˚C for future use.

Erythrocyte Lysate Preparation

1. Collect blood in vacutainers containing heparin or sodium citrate as an anticoagulant. Centrifuge at 3,000 x g for 10-15 minutes at 2-8 ˚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 2-8 ˚C.
5. Collect the clarified supernatant. Store on ice if you intend to immediately assay for glutathione, or freeze at ≤ -70 ˚C for future use.

Whole Blood Lysate Preparation

1. Collect blood in tubes containing heparin or sodium citrate as an 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 2-8 ˚C.
4. Collect the clarified supernatant. Store on ice if you intend to immediately assay for glutathione. Freeze at ≤ -70 ˚C for future use.

Urine, Plasma, and Saliva Lysate 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 2-8 ˚C.
3. Collect the clarified supernatant. Store on ice if you intend to immediately assay for glutathione. Freeze at -70 ˚C for future use.

ASSAY PROTOCOL

It is recommended that all standards, samples, and controls be assayed in triplicate.

Total Glutathione Assay

1. Prepare all reagents as directed in previous section.
2. Immediately prior to assay, dilute each experimental sample 1:10 with 1X Assay Buffer. Some biological specimens such as whole blood, liver, or red blood cells may need to be diluted 1:20, 1:40, or more.
3. Set up the GSSG 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.
   b. Add 50 µL of the 4.0 µM 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 µL from wells A1, A2, and A3 to wells B1, B2, and B3, respectively. Mix well by pipetting 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.
4. Add 50 µL of diluted experimental sample to the wells in Columns 4 -12, as needed.
   Note: *It may be necessary to make serial dilutions of your samples to obtain a satisfactory change in absorbance readings.*
5. 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.
6. Using a multi-channel pipetor, add 150 µL of freshly-prepared Glutathione Reductase Reaction Mix to each well.
7. 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 1.0 µL of 2M 4-Vinylpyridine per 50 µL of sample and per 50 µL of 4.0 µM GSSG. Incubate for 1 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 Curve as described above in the Total Glutathione Assay Protocol.
3. Serially dilute your 4-Vinylpyridine-treated experimental samples as described above in the Total Glutathione Assay Protocol.
4. Follow steps 5, 6, and 7 as described for the Total Glutathione Assay.

DATA INTERPRETATION

Determination of Total Glutathione Concentration

1. At each time point, take the average of the triplicate absorbance readings for each standard, sample, and background at each time point.
2. Plot the average of each standard, sample, and background absorbance (405 nm) versus the incubation time and determine the slope from the linear portion of each curve.
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.
5. Compare the net slopes of the experimental samples with those of the standard curve to determine the pmoles of GSSG (equivalent to total glutathione) for each experimental sample.

Determination of Oxidized Glutathione Concentration

1. Follow the procedure described in the Total Glutathione Concentration protocol to generate the GSSG Standard Curve 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 standards curve 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.

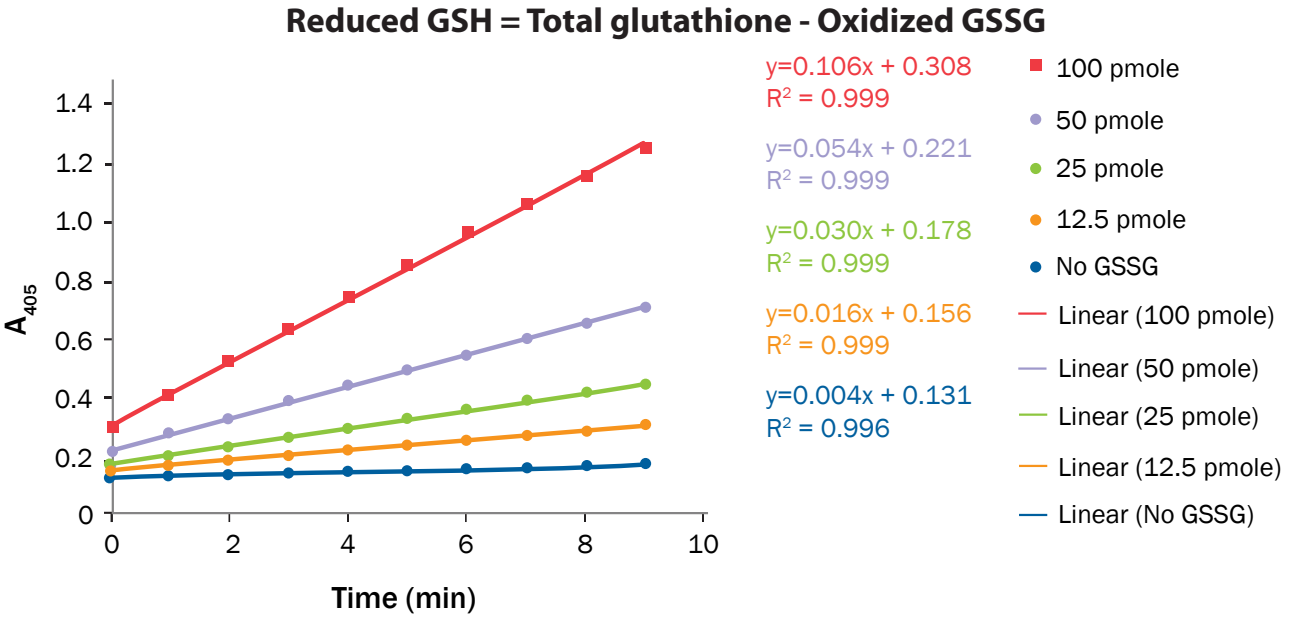


Figure 1. Plot of absorbance at 405 nm versus incubation time as a function of pmoles of GSSG/well.

DATA INTERPRETATION *CONTINUED*

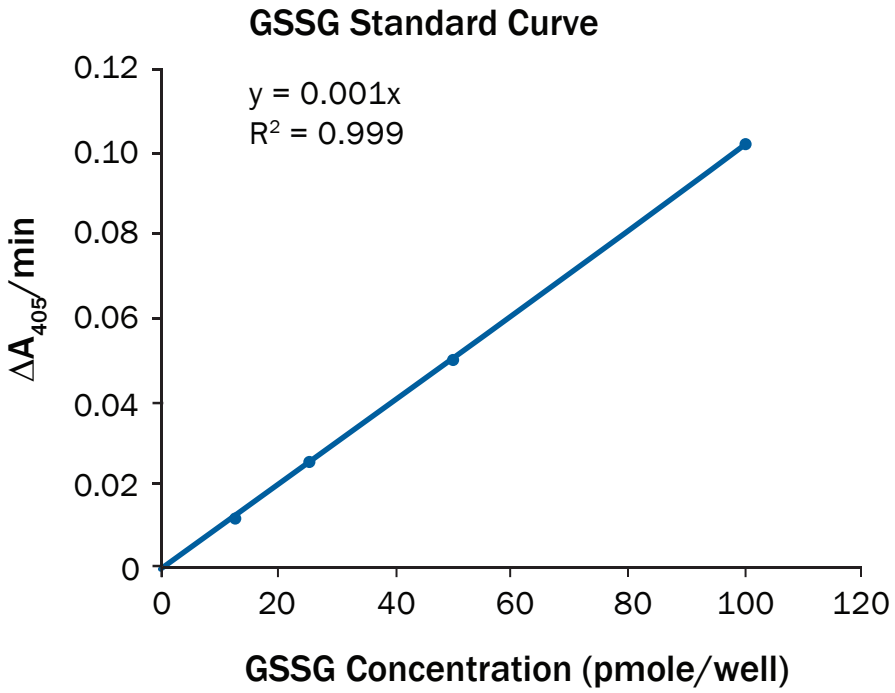


Figure 2. Rate of increase in the absorbance at 405 nm as a function of pmole/well GSSG.

TROUBLESHOOTING

Problem	Cause	Action
High CVs.	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 Reaction Mix.
	NADPH is oxidized.	Contact Bio-Techne Technical Services.
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 rerun assay.

REFERENCES

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