TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

HT Glutathione Assay Kit

Colorimetric assay for total, reduced and oxidized glutathione.

Sufficient reagents for 384 tests.

Catalog# 7511-100-K

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I. Background

Increased oxidative damage of biomolecules (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, the major intracellular non-protein thiol, is an important protector against free radical damage by providing reducing equivalents for several key antioxidant enzymes and also by scavenging hydroxyl radicals and nascent oxygen. High reduced Glutathione levels are associated with fewer numbers of illnesses, higher levels of self-rated health, lower cholesterol, lower body mass index, and lower blood pressures 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 1-10 mM in cells and is in the micromolar range in plasma.

Features of the Kit:

-Suitable for mammalian cells, tissue, blood, plasma and other bodily fluids.
-Contains sufficient reagents to assay 384 data points or to determine Glutathione in:
A) 123 experimental samples, each performed in triplicate, plus one Glutathione standard curve;
B) 108 experimental samples, each performed in triplicate, plus 4 Glutathione standard curves;
C) 88 experimental samples, each performed in triplicate, plus 8 Glutathione standard curves.

II. Principle of the Assay

Trevigen's **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). As shown in Figure 1 (below), 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, and the mixed disulfide, GSTNB, that 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 in turn 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.

III. Precautions and Limitations

A. For research use only. Not for use in diagnostic procedures.
B. The physical, chemical and toxicological properties of the kit components have not been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

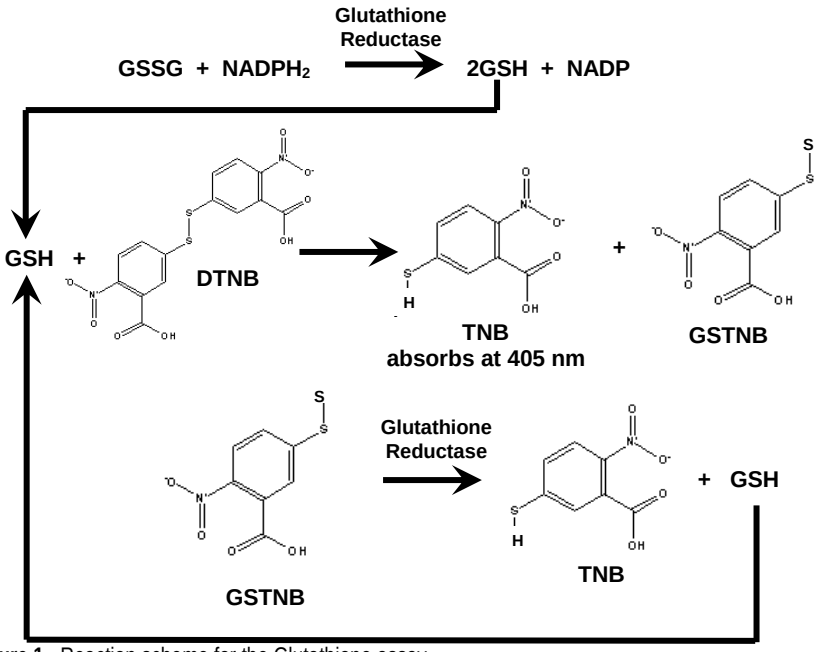

Figure 1. Reaction scheme for the Glutathione assay.

IV. Materials Supplied

Cat#	Component	Amount	Storage
7511-100-01	Glutathione Reductase	80 µl	4°C
7511-100-02	25X Assay Buffer	12 ml	4°C
7511-100-04	Reaction Mix	8 bottles	4°C
7511-100-05	96 well plates	8	4°C
7511-100-06	4 µM GSSG	2.5 ml	4°C

V. Reagents/Equipment Required But Not Supplied

Equipment
96 well plate reader
Multichannel pipetor
Pipetor
Pipette tips
Centrifuge (for cell lysis)

Reagents
High quality, double-distilled H_2O
Metaphosphoric acid (Aldrich Cat# 23,927-5)
4-Vinylpyridine (Aldrich Cat# V3204-5ML)
Reagent alcohol

VI. Reagent Preparation

1. 1X Assay Buffer

Prior to each experiment, prepare the necessary amount of 1X Assay Buffer by diluting the 25X Assay Buffer (Cat# 7511-100-02) with dH_2O.

2. Reaction Mix

Reconstitute one or more bottles of Reaction Mix (Cat # 7511-100-04) with 8 ml of dH_2O per bottle. Vortex each bottle until the contents completely dissolve (~10 sec). **Immediately before use in the assay, vortex the vial of Glutathione Reductase** (Cat# 7511-100-01) and add 10 µl 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 Cat# 23,927-5, not provided) in dH_2O.

4. GSSG

The GSSG (Cat# 7511-100-06) is provided at a concentration of 4 µM (4 pmoles/µl). Store any unused portion at 4˚C.

5. 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 (Aldrich Cat# V3204-5ML, not provided) 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 you use 4-vinylpyridine within 1 month of purchase and store at -20°C.

VII. Sample Preparation

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

A. 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.

2. Suspend the pellet with 500 µl of 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 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.

B. 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 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.

C. 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.

D. 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.

E. 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 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.

VIII. Assay Protocol

A. Total Glutathione Assay

1. **Immediately prior to assay, dilute each experimental sample 10-fold with 1X Assay Buffer.** Some biological specimens such as whole blood, liver, or red blood cells may need to be diluted 20-fold, 40-fold, or more.

2. 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 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 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.

3. 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. A sample dilution scheme is shown in Figure 2.

4. 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.

5. Using a multichannel pipetor, add 150 µl of freshly-prepared Reaction Mix to each well (see Section **VI.2**).

6. 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 µl of 2M 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 **VIII. A.2**).

3. Serially dilute your 4-vinylpyridine-treated experimental samples as described above in the total glutathione assay protocol (Step **VIII. A.3**).

4. Follow steps 4, 5, and 6 as described above for the total glutathione assay.

IX. Data Interpretation

For your convenience, a calculation worksheet (MS Excel required) is provided: http://www.trevigen.com/docs/1427836056.7511-100-k_calculation_worksheet.xls.

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 standards 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

	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.

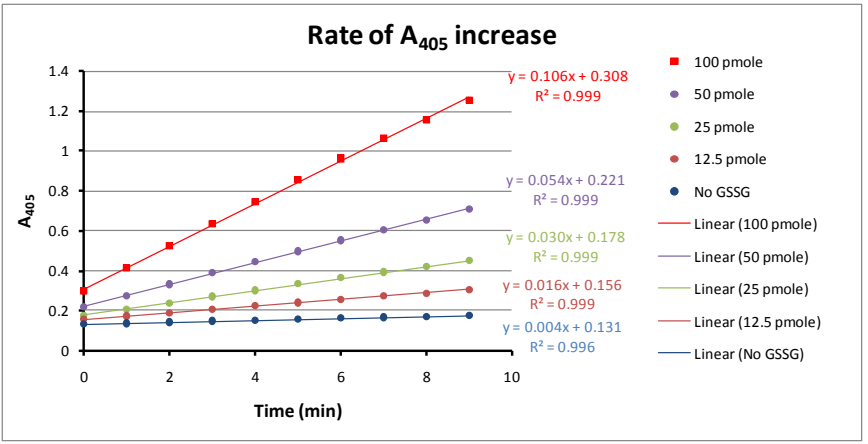

Figure 3. Plot of absorbance at 405 nm versus incubation time as a function of pmoles of GSSG/well.

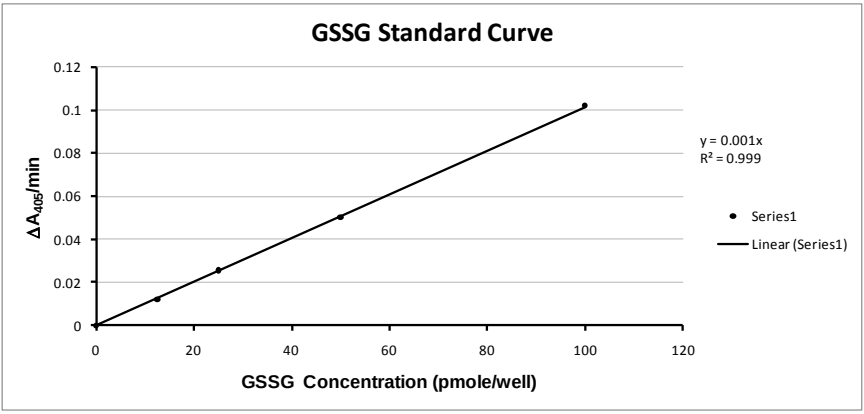

Figure 4. Rate of increase in the absorbance at 405 nm as a function of pmole/well GSSG.

X. 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
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3. Julius M, Lang CA, Gleiberman L, Harburg E, DiFranceisco W, Schork A. 1994. Glutathione and morbidity in a community-based sample of elderly., *J Clin epidemiol.* **47**: 1021-1026.
4. Mytilineou C, Kramer BC, Yabut JA. 2002. Glutathione depletion and oxidative stress. *Parkinsonism Relat Disord*. **8**:385-387.

XI. Troubleshooting

Problem	Cause	Solution
Erratic within sample values	Inconsistent pipetting technique	Reproducible pipetting is absolutely required for consistent results
Erratic within sample values	Reaction Mix incompletely solubilized	Periodically vortex the Reaction Mix over a 15 minute period.

Problem	Cause	Solution
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 Trevigen
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

XII. Related Products Available from Trevigen

Catalog #	Description	Size
4870-500-6	10X PBS, pH 7.4	6 X 500 ml
7500-100-K	Superoxide Dismutase Assay Kit	100 tests
7501-500-K	HT Superoxide Dismutase Assay Kit	480 tests
7510-100-K	Glutathione Reductase Assay Kit	100 tests
7513-500-K	HT Glutathione Reductase Assay Kit	480 tests
7512-100-K	HT Glutathione Peroxidase Assay Kit	480 tests

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