

HT Glutathione Peroxidase Assay Kit

**Spectrophotometric assay kit for the
quantitation of Glutathione Peroxidase in
cells, erythrocytes, blood, and tissues.**

Sufficient reagents for:

**480 Tests-96 well Format
100 Tests-cuvette Format**

Cat# 7512-100-K

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100 Tests-Cuvette Format**

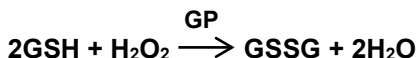
Cat# 7512-100-K

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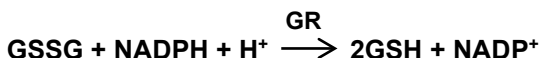
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I. Introduction

Glutathione peroxidase is a tetramer of four identical subunits, with a molecular weight of 84,000. It requires selenium as a cofactor and contains a selenocysteine amino acid residue in the active site of each monomer that participates in the actual mechanism of the enzyme. Glutathione peroxidase (GP) is found in mammalian cells and helps to prevent lipid peroxidation of cell membranes by consuming free peroxide in the cell. The enzyme catalyzes the following reaction:



Glutathione Reductase (GR) then reduces the oxidized glutathione to complete the cycle:



Where GSH represents reduced monomeric glutathione, and GSSG represents oxidized glutathione. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A₃₄₀ is directly proportional to the Glutathione Peroxidase activity in the sample. Trevigen's HT Glutathione Peroxidase Assay Kit can be used to measure glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the Glutathione Peroxidase Assay Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

Catalog Number	Component	Amount Provided	Storage Temperature
7512-100-01	Glutathione Peroxidase	800 µl	-20°C
7512-100-02	10X Assay Buffer	20 ml	-20°C
7512-100-03	GSH + NADPH	10 vials	-20°C
7512-100-04	Glutathione Reductase	1.1 ml	-20°C
7512-100-05	Cumene Hydroperoxide	12 ml	-20°C
7512-100-06	96-well plates	5 plates	R.T.

IV. Materials/Equipment Required But Not Supplied

Reagents:

- A. Samples to be tested.
- B. PBS, pH 7.4
- C. Distilled water
- D. Phenylmethyl Sulfonyl Fluoride (PMSF) and other protease inhibitors
- E. Peroxide-free Triton X-100 or Nonidet P-40 for cell extract preparation.

Disposables:

- A. 1 - 200 µl and 100-1000 µl pipette tips
- B. 1.5 ml tubes

Equipment:

- A. Micropipettes
- B. Sonicator or homogenizer
- C. Multichannel pipettors: 1 - 50 µl and 50 – 200 µl
- D. 96-well plate reader with 340 nm filter or spectrophotometer capable of reading at 340 nm
- E. Microcentrifuge

V. Reagent Preparation

1. 10X Assay Buffer (Cat# 7512-100-02)

This solution is used in the assay and to prepare your samples. Dilute **1:10** to **1X** with dH₂O before use. Store any unused portion at 4°C. The 1X Assay Buffer is stable at 4°C for up to 2 weeks. Note that 1X Assay Buffer contains 0.1 mg/ml BSA and this should be taken into account when calculating the protein concentration of your samples.

2. GSH + NADPH (Cat# 7512-100-03)

Each vial of lyophilized GSH + NADPH contains sufficient reagent for 50 assays in a microwell format or 10 assays in a cuvette format. Bring the number of vials required to room temperature and add 110 µl of 1X Assay Buffer to each vial. Swirl contents gently to dissolve and store on ice. The reconstituted reagent should be used within 4 hours. Discard any unused portion after use. Do not refreeze.

3. 10X Reaction Mix

Make a 10X Reaction Mix (sufficient for 50 assays in a microwell format or 10 assays in a cuvette format) as follows:

Glutathione Reductase (Cat# 7512-100-04)	110 µl
Reconstituted GSH + NADPH	110 µl
1X Assay Buffer	880 µl

Mix well and use within 4 hours.

4. Cumene Hydroperoxide (Cat# 7512-100-05)

Thaw the cumene hydroperoxide (Cat# 7512-100-05) and bring to room temperature. Aliquot into 1.1 ml portions and freeze at -20°C. Each aliquot is sufficient for 50 assays in a microwell format or 10 assays in a cuvette format.

VI. Sample Preparation

A. Preparation of Cell Extracts

1. **Non-adherent cells:** Centrifuge 2×10^6 to 1×10^7 non-adherent cells at 400 x g for 10 minutes at 4°C. Discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge at 10,000 x g for 10 sec at 4°C. Discard the supernatant. Suspend the cell pellet in 5 pellet volumes of cold 1X Assay Buffer containing 0.4 mM PMSF, other protease inhibitors, and 1% Triton X-100 (peroxide-free).
2. **Adherent cells:** Wash the adherent cells with 1X PBS. Adherent cells may be harvested by scraping in 5 ml of ice-cold 1X PBS. Transfer to a prechilled 15 ml tube. Centrifuge at 400 x g for 10 minutes at 4°C and discard the supernatant. Resuspend the cell pellet in 1 ml ice-cold 1X PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge at 10,000 x g for 12 sec at 4°C. Discard the supernatant. Resuspend the cell pellet in 5 pellet volumes of ice-cold 1X Assay Buffer containing 0.4 mM PMSF, other protease inhibitors, and 1% Triton X-100 (peroxide-free).
3. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
4. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10-20 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice.
5. Determine the protein concentration of the cleared cell lysate. Snap-freeze the cleared cell extract in small aliquots and store at -80°C. Avoid repeated freezing and thawing of the extract. The frozen cell extracts will be stable for 1 month.

B. Preparation of Plasma

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge at 1,500 x g for 10 minutes at 4°C. Pipette off the upper yellow plasma layer without disturbing the white buffer layer (the white interface between the pelleted red blood cells and the plasma).

3. Store the plasma on ice for up to 4 hours, or freeze in small aliquots at -80°C. The frozen samples will be stable for at least 1 month.

C. Preparation of Red Blood Cell Lysates

1. Follow the directions for preparing plasma, above. Remove the buffy coat and discard.
2. Wash the red blood cells with 1X PBS at 4°C. Centrifuge at 1500 x g for 10 minutes at 4°C. Discard the supernatant. Repeat this step once more.
3. Lyse the red blood cells in 4-10 volumes of 4°C deionized water by repeated gentle vortexing or mixing over a 10 minute period.
4. Centrifuge at 10,000 x g for 15 minutes at 4°C. Collect the supernatant.
5. Determine the protein concentration of the erythrocyte lysate. Store on ice for up to 4 hours, or freeze in small aliquots at -80°C. The frozen samples will be stable for at least one month.

D. Preparation of Tissue Homogenates

1. Prior to dissection, perfuse the tissue with 1X PBS plus 0.16 mg/ml heparin to remove blood components and clots.
2. Homogenize the tissue in 5-10 ml per gram of tissue of cold 1X Assay Buffer containing 0.4 mM PMSF, other protease inhibitors, and 1% Triton X-100 (peroxide-free).
3. Centrifuge at 10,000 x g for 10-20 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice
4. Determine the protein concentration of the cleared tissue lysate.
5. **Alternative protocol:** Mince the tissue in cold 1X PBS and make a single cell suspension by forcing the tissue through a stainless steel wire mesh screen using a pestle. Centrifuge the single cell suspension at 1,000 x g for 10 minutes at 4°C. Discard the supernatant. Resuspend the pellet in 5 pellet volumes of ice-cold 1X Assay Buffer containing 0.4 mM PMSF, other protease inhibitors, and 1% Triton X-100 (peroxide-free).
6. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
7. Microcentrifuge the disrupted tissue single cell suspension at 10,000 x g for 10-20 minutes at 4°C to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice (If supernatant is cloudy, repeat centrifugation).
8. Determine the protein concentration of the cleared tissue lysate.

9. Store the clarified tissue extracts on ice and assay immediately, or snap-freeze the cleared tissue extract in small aliquots and store at -80°C. Avoid repeated freezing and thawing of the extract. The frozen cell extracts will be stable for 1 month.

VII. Glutathione Peroxidase Assay Protocol

A. 96-Well Plate Format

1. Set up your plate reader to measure absorbance at 340 nm every 30 seconds or 1 minute. Accuracy and consistency of results is dependent on maintaining a constant temperature. Set the plate chamber of your instrument to 25°C if possible. Zero your readings with respect to 200 µl of 1X Assay Buffer in one well.
2. Plan your experiment to measure each sample in triplicate and at different volumes or amount of protein. Some samples, particularly erythrocyte lysates, may have to be diluted significantly with 1X Assay Buffer to achieve a reasonable rate of decrease in absorbance at 340 nm. Note that the final volume of the reaction is 200 µl.
3. Set up the following reactions in a 96 well plate (per well):

1X Assay Buffer:	140 µl
10X Reaction Mix:	20 µl
Glutathione Peroxidase (cat# 7512-100-01) or sample	20 µl

4. Initiate the reactions by quickly adding 20 µl of Cumene Hydroperoxide to each well using a multichannel pipettor, and pipette up-and-down 3x to mix.
5. Immediately begin measuring absorbance at 340 nm every 30 seconds or 1 minute over a 10-15 minute period.
6. **Controls:** Include a background set of tubes where 20 µl of 1X Assay Buffer is added instead of sample or Glutathione Peroxidase. The rate of decrease of absorbance at 340 nm in the background is subtracted from that of the samples or standard to obtain the net rate of decrease of absorbance at 340 nm for the calculation of Glutathione Peroxidase activity in your samples.

B. Cuvette Format

1. Set up your spectrophotometer to measure absorbance at 340 nm every 30 seconds or 1 minute. Zero the instrument with 1 ml of 1X Assay Buffer. Accuracy and consistency of results is dependent on maintaining a constant temperature. If possible, set the cuvette chamber of your instrument to 25°C.
2. Set up the following reaction in a 1.5 ml tube:

1X Assay Buffer:	700 µl	<u>Order of Addition</u>
		1

10X Reaction Mix:	100 μ l	2
Glutathione Peroxidase or sample	100 μ l	3
Cumene Hydroperoxide	100 μ l	4

- Mix well by inverting the tube several times. Transfer the contents to a cuvette and immediately begin measuring absorbance at 340 nm every 30 seconds or 1 minute over a 5-10 minute period.

VIII. Data Interpretation.

For your convenience, excel calculation worksheets are provided (MS excel required):

(i) http://www.trevigen.com/docs/1427922552.7512-100-k_plate_assay_calculation_worksheet.xls

(ii) http://www.trevigen.com/docs/1427922531.7512-100-k_cuvette_assay_calculation_worksheet.xls.

A. Glutathione Peroxidase Activity Determination

- Calculate the mean absorbance at each time point of your triplicates for your experimental samples, for the Glutathione Peroxidase Standard, and for the background.
- Plot the mean absorbance versus time. A representative standard curve is shown in **Figure 1**:

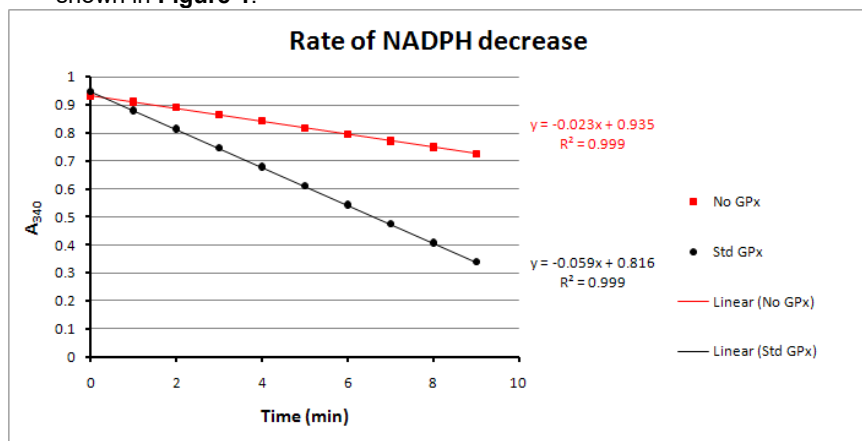


Figure 1: Graph of absorbance at 340 nm versus incubation time of the Glutathione Peroxidase standard and of the background absorbance. The net $\Delta A_{340}/\text{min}$ of the Glutathione Peroxidase standard should be about $(-0.090 \text{ to } -0.035) \text{ min}^{-1}$ for the cuvette, and $(-0.057 \text{ to } -0.02) \text{ min}^{-1}$ for the plate format.

- Determine the slope from a linear portion of the curve for your sample, the Glutathione Peroxidase standard, and the background. Express the results as the change in absorbance per minute ($\Delta A_{340}/\text{min}$)
- Subtract the $\Delta A_{340}/\text{min}$ for the background from that of the samples.

- One Unit of Glutathione Peroxidase is defined as the amount of enzyme that will cause the oxidation of 1 nmole of NADPH to NADP⁺ per minute at 25°C. The reaction rate can be calculated knowing the extinction coefficient of NADPH, which is 0.00622 $\mu\text{M}^{-1}\text{cm}^{-1}$. Since the path length of the samples in the wells is 0.61 cm, the extinction coefficient is modified to 0.00379 μM^{-1} . Calculate the Glutathione Peroxidase activity in your samples from the following equations (where Y is the volume of your sample in the reaction):

96-Well Plate Format:

$$\text{Glutathione Peroxidase Activity} = \frac{\Delta A_{340}/\text{min}}{0.00379 \mu\text{M}^{-1}} \times \frac{0.2 \text{ ml}}{Y \text{ ml}} \times \text{Sample Dilution}$$

$$= \text{nmole/min/ml} = \text{Units/ml}$$

Cuvette Format:

$$\text{Glutathione Peroxidase Activity} = \frac{\Delta A_{340}/\text{min}}{0.00622 \mu\text{M}^{-1}} \times \frac{1.0 \text{ ml}}{Y \text{ ml}} \times \text{Sample Dilution}$$

$$= \text{nmole/min/ml} = \text{Units/ml}$$

IX. References

- Ozdemir G, Ozden M, Maral H, Kuskay S, Cetinalp P, Tarkun I. Malondialdehyde, glutathione, glutathione peroxidase and homocysteine levels in type 2 diabetic patients with and without microalbuminuria. *Ann Clin Biochem.* 2005, **42**:99-104.
- Prasad NR, Menon VP, Vasudev V, Pugalendi KV. Radioprotective effect of sesamol on gamma-radiation induced DNA damage, lipid peroxidation and antioxidants levels in cultured human lymphocytes. *Toxicology.* 2005, **209**:225-35.
- Sindhu RK, Ehdaie A, Farmand F, Dhaliwal KK, Nguyen T, Zhan CD, Roberts CK, Vaziri ND. Expression of catalase and glutathione peroxidase in renal insufficiency. *Biochim Biophys Acta.* 2005, **1743**:86-92.
- Szelestei T, Bähring S, Wagner Z, Aydin A, Molnar GA, Kocsis B, Nagy J, Wittmann I. Serum levels of L-arginine analogues and glutathione peroxidase and catalase gene variants in Type 2 diabetes mellitus patients. *Diabet Med.* 2005, **22**:356-7.
- Chu FF, Esworthy JH, Doroshow JH. Role of Se-dependent glutathione peroxidases in gastrointestinal inflammation and cancer. *Free Rad Biol Med.* 2004. **36**:1481-95.

X. Troubleshooting

PROBLEM	CAUSE	SOLUTION
Erratic values. Poor reproducibility of triplicates	Poor pipetting technique	Depress plunger of your pipettor all the way. Fill with solution. Depress to first stop. Fill with solution. Depress to first stop. Repeat as often as necessary.
	Bubbles in well	Avoid making bubbles.
PROBLEM	CAUSE	SOLUTION
No decrease in absorbance observed in sample wells or Glutathione Peroxidase standard.	Enzyme activity in the samples is too low.	Increase volume and/or concentration of your samples.
	Failure to add cumene hydroperoxide	Add cumene hydroperoxide to wells.
Initial absorbance is below 0.5 or the absorbance decreases very rapidly	Glutathione Peroxidase activity is too high in your samples.	Dilute your samples with 1X Assay Buffer and reassay
Initial absorbance is less than 0.1 and there is no decrease in absorbance	Failure to add GSH + NADPH to 10X Reaction Mix	Add GSH + NADPH to 10X Reaction Mix
Initial absorbance in sample wells is greater than 1.5	Sample is too concentrated	Dilute your sample with 1X Assay Buffer and re-assay

XI. Related Products Available From Trevigen

<u>Catalog #</u>	<u>Description</u>	<u>Size</u>
4870-500-6	10X PBS, pH 7.4	6 X 500 ml
7500-100-K	Superoxide Dismutase Assay Kit	1 Kit
7510-100-K	Glutathione Reductase Assay Kit	1 Kit
7511-100-K	Glutathione Assay Kit	1 Kit

XII. Appendix

A. Reagent composition:

- 1X PBS (pH 7.4):** 7.5 mM Na_2HPO_4 , 2.5 mM NaH_2PO_4 , 145 mM NaCl.
- Glutathione Peroxidase (Cat# 7512-100-01):** Bovine erythrocyte glutathione peroxidase provided at a concentration sufficient to generate a net $\Delta A_{340}/\text{min}$ of about -0.068/min in the cuvette assay and -0.044/min in the plate assay.
- 10X Assay Buffer (Cat# 7512-100-02):** Proprietary buffer solution containing 1 mg/ml BSA.

4. **Glutathione + NADPH (Cat# 7512-100-03):** A lyophilized reagent which, when reconstituted with 110 μ l of 1X Assay Buffer, yields Glutathione and NADPH concentrations of 100 mM and 10 mM, respectively.
5. **Glutathione Reductase (Cat# 7512-100-04):** Provided at a concentration of 0.025 Unit/ μ l.
6. **Cumene Hydroperoxide:** Provided at 5 mM in 1X Assay Buffer.