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Stressgen

> Glutathione Peroxidase Activity Kit

Catalog # 900-158

Sufficient Reagents for 480 tests in 5 x 96-well plates
For use with mammalian cells, tissues, erythrocytes, and plasma.

All reagents should be stored at -20˚C.

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

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

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

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Introduction

Assay Designs' Glutathione Peroxidase Activity Kit is a complete kit for measuring the activity of glutathione-dependent peroxidases in cells, tissues, erythrocytes and plasma.

Glutathione Peroxidase is a tetramer of four identical subunits, with a molecular weight of 84,000 daltons. 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:

$$2GSH + H_2O_2 \xrightarrow{GP} GSSG + 2H_2O$$

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

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

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 (A340). The rate of decrease in the A340 s directly proportional to the Glutathione Peroxidase activity in the sample.

Principle

1. Reaction mix, Glutathione Peroxidase, and samples are added to the appropriate wells of a 96-well plate.

2. Cumene Hydroperoxide is added to the wells to initiate the reaction.

3. The plate is transferred to a plate reader and absorbance readings are taken at 340 nm every 30 seconds or 1 minute for 10 to 15 minutes.

Materials Supplied

1. Clear Microtiter Plate
   Five Plates of 96 Wells, Catalog No. 80-1639
   Clear uncoated solid plates.

2. 10X Assay Buffer
   20 mL, Catalog No. 80-1662

3. GSH + NADPH
   10 vials, Catalog No. 80-1661

4. Glutathione Reductase
   1.1 mL, Catalog No. 80-1665

5. Glutathione Peroxidase
   800 µL, Catalog No. 80-1664
   Glutathione Peroxidase in a 50% glycerol storage buffer

6. Cumene Hydroperoxide
   12 mL, Catalog No. 80-1663

Storage

All components of this kit are stable at -20°C. All kit components are stable at their recommended storage temperatures until the kit expiration date.

Materials Needed but Not Supplied

1. PBS pH 7.4
2. Distilled water
3. Protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), Sigma P7626 or equivalent
4. Peroxide free Triton X-100 or Nonidet P-40 for cell extract preparation.
5. Microtubes, 0.5 and 1.5 mL
6. 15 mL conical tubes (adherent and suspension cell preparation)
7. 50 mL conical tubes (tissue preparation)
8. Precision pipettes for volumes between 1-200 µL and 100 to 1000µL
9. Multichannel pipettor for volumes between 1 - 50 µL and 50 µL – 200 µL
10. Microplate reader or spectrophotometer capable of reading at 340 nm and taking readings every 30 seconds or 1 minute for ten to 15 minutes and exporting data to an Excel spreadsheet.
11. Microcentrifuge for processing samples
12. 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.

Reagent Preparation

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.

Store the reconstituted NADPH + GSH on ice.

Thaw Glutathione Reductase Stock on ice. Keep on ice while in use.

1. **Assay Buffer**
   Dilute the 10X Assay Buffer to 1X (1:10) with distilled water. 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**
   Each vial of lyophilized GSH + NADPH contains sufficient reagent for 50 wells. 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. **Reaction Mix**
   Make a 10X Reaction Mix (sufficient for 50 wells) as follows:

Glutathione Reductase	110 µL
Reconstituted GSH + NADPH	110 µL
1X Assay Buffer	880 µL

   Mix well and store on ice until added to the wells. Reaction Mix should be used within 4 hours of preparation.

4. **Cumene Hydroperoxide**
   Thaw the cumene hydroperoxide and bring to room temperature. Aliquot into 1.1 mL portions and freeze at -20°C. Each aliquot is sufficient for 50 wells. Note that the Cumene Hydroperoxide should be used within 1 hour at room temperature. Store on ice if in use for longer periods of time.

Sample Handling

A. Preparation of Cell Extracts

Samples must be kept on ice to maintain enzyeme activity.

1. **Non-adherent cells:** Centrifuge 2 x 10^6 to 1 x 10^7 non-adherent cells at 400 x g for 10 minutes at 4°C. Discard the supernatant. Suspend the cell pellet in1mL of ice-cold 1X PBS and transfer to a prechilled 1.5 mL microtube. Centrifuge at 10,000 x g for 10 seconds 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 pre chilled 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 pre chilled 1.5 mL microtube. Centrifuge at 10,000 x g for 12 seconds 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 pre chilled 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. Pipet 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.

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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 sus pension 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.

Assay Procedure

1. Set up your plate reader to measure absorbance at 340 nm every 30 seconds or 1 minute. Include a 10 second orbital shake prior to the initial read. Accuracy and consistency of results is dependent on maintaining a constant temperature. Set the plate chamber of your instrument to 25°C if possible. Blank 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 dilutions or amounts 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, sample, or control	20 µL

   Note: The Glutathione Peroxide is provided to serve as a positive control to ensure the assay is working. It should not be used to construct a calibration curve to measure Glutathione Peroxidase concentration in samples.

4. Initiate the reactions by quickly adding 20 µL of Cumene Hydroperoxide to each well using a mutichannel pipettor.

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 wells 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.

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.

Thaw the Glutathione Peroxidase stock on ice. Keep on ice while in use.

Wells containing Glutathione Peroxidase Standard must be well-mixed. Avoid creating bubbles.

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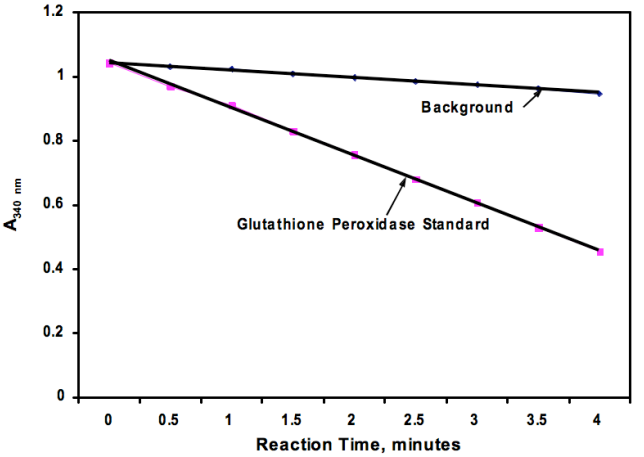
Calculation of Results

A. Determination of Glutathione Peroxidase Activity.

1. Calculate the mean absorbance at each time point of the triplicate values for the samples, Glutathione Peroxidase Standard, and background.

2. Plot the mean absorbance versus time. A representative standard curve is shown in Figure 1:



3. Determine the slope from a linear portion of the curve for the sample, Glutathione Peroxidase standard, and background. Express the results as the change in absorbance per minute (ΔA340/min).

4. Subtract the ΔA340/min for the background from that of the samples.

5. 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 µM -1cm-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 samples):

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

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

Trouble Shooting

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
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 deceases 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 reassay

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Notes

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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.

MSDS (Material Safety Data Sheet) available online

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.
5777 Hines Drive
Ann Arbor, Michigan
USA

tel: 734.668.6113 or 800.833.8651
fax: 734.668.2793
info@assaydesigns.com
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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