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# OxisResearch™

A Division of OXIS Health Products, Inc.

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## BIOXYTECH<sup>®</sup> GPx-340™

Colorimetric Assay for Cellular Glutathione Peroxidase  
For Research Use Only. Not For Use In Diagnostic Procedures.  
Catalog Number 21017

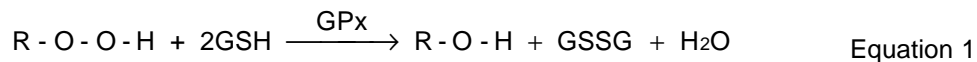
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### INTRODUCTION

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#### The Analyte

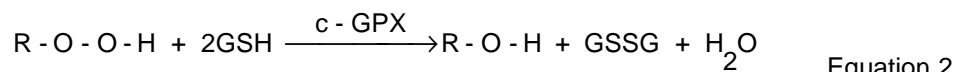
Cellular glutathione peroxidase (c-GPx, EC 1.11.1.9) is a member of a family of GPx enzymes whose function is to detoxify peroxides in the cell.<sup>1</sup> Because peroxides can decompose to form highly reactive radicals, the GPx enzymes play a critical role in protecting the cell from free radical damage, particularly lipid peroxidation. The GPx enzymes catalyze the reduction of H<sub>2</sub>O<sub>2</sub> to water and organic peroxides (R-O-O-H) to the corresponding stable alcohols (R-O-H) using glutathione (GSH) as a source of reducing equivalents:



With the exception of phospholipid-hydroperoxide GPx, a monomer, all of the GPx enzymes are comprised of 4 identical subunits (monomer Mr 22-23 kDa). Each subunit contains a molecule of selenocysteine in the enzyme active site. The selenocysteine is thought to participate directly in electron donation to the peroxide substrate and become oxidized in the process. The enzyme then uses glutathione as an electron donor to regenerate the reduced form of the selenocysteine.<sup>1</sup> The GPx enzymes accept a wide variety of organic peroxides as substrates. However, with the exception of phospholipid hydroperoxide GPx and perhaps pl- GPx, the enzymes exhibit a strong preference for glutathione as a source of reducing equivalents. Phospholipid-hydroperoxide GPx (Mr 19 kDa) is the only enzyme with significant activity on esterified phospholipids and cholesterol in membranes.

#### Principles Of The Procedure

The GPx-340™ assay is an indirect measure of the activity of c-GPx.<sup>7</sup> Oxidized glutathione (GSSG), produced upon reduction of an organic peroxide by c-GPx, is recycled to its reduced state by the enzyme glutathione reductase (GR):



The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm (A<sub>340</sub>) providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 M<sup>-1</sup> cm<sup>-1</sup> at 340 nm. To assay c-GPx, a cell or tissue homogenate is added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is initiated by adding the substrate, tert-butyl hydroperoxide and the A<sub>340</sub> is recorded. The rate of decrease in the A<sub>340</sub> is directly proportional to the GPx activity in the sample.

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## REAGENTS

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### **Materials Provided (for 100 tests)**

- NADPH Reagent                   β-nicotinamide-adenine dinucleotide phosphate (reduced), Glutathione, and Glutathione reductase. Lyophilized and when reconstituted with 7.5 mL Assay Buffer, each of the 5 vials provide for 20 tests.
- Assay Buffer                       pH 7.6, 120 mL
- tert-Butyl Hydroperoxide       In water, 2.0 mL

### **Materials Required But Not Provided**

- Spectrophotometer, preferably equipped with a temperature-controlled cuvette chamber, capable of measuring the absorbance at 340 nm.
- Spectrophotometric cuvettes with a 1 cm path length.
- Adjustable pipettors, 10 μL to 1 mL with disposable pipette tips.
- 10 mL glass or plastic pipettes, capable of accurately measuring 7.5 mL.
- Beakers, flasks or small bottles (20-50 mL capacity) for Assay Buffer and tert-Butyl Hydroperoxide.
- Deionized water
- Bioxytech® Cellular Glutathione Peroxidase Control, Catalog # 27617Q.

### **Warnings and Precautions**

Use established laboratory precautions when handling or disposing any chemical contained in this product. Refer to the Material Safety Data Sheet for risk, hazard, and safety information.

### **Reagent Storage and Handling**

All reagents should be stored at 2-8 °C and are stable, if unopened, until the expiration date.

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## PROCEDURE

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### **Reagent Preparation**

#### **NADPH**

Add 7.5 mL of Assay Buffer to the desired number of NADPH Reagent vials (20 tests per vial). Bring the reagent to assay temperature (recommended 23-25°C). If capped and protected from light, the vial can then be kept at ambient temperature (21-27 °C) for a period of 9 hours, or at 37 °C for 3 hours, without a significant effect on c-GPx assay performance. Do not freeze the reconstituted reagent.

#### **Assay Buffer**

Estimate the amount of Assay Buffer required to complete the intended number of samples for the day. Pour that amount of Buffer from the kit bottle into a separate container, preferably one with a cap, and bring to assay temperature. Discard the unused Buffer at the end of the day; it is not recommended that unused Assay Buffer be returned to the original kit bottle.

#### **Tert-Butyl Hydroperoxide**

Dilute the tert-Butyl Hydroperoxide substrate to 1/10,000 in deionized water, and set in a closed container at the assay temperature. The working substrate solution may be kept at assay temperature for an entire working day. It should be made fresh each day.

Example dilution protocol:

Intermediate dilution, 1/100 dilution of tert-Butyl Hydroperoxide:  
0.05 mL tert-Butyl Hydroperoxide stock  
+ 4.95 mL deionized water  
Working Substrate, 1/10,000 dilution of tert-Butyl Hydroperoxide,  
1/100 dilution of intermediate:  
0.2 mL intermediate dilution  
+ 19.8 mL deionized water

### **Sample Preparation**

Thaw, and place on ice.

Note: Please read the appropriate **NOTES** sections before starting sample preparation procedure.

### **Assay**

1. Turn on spectrophotometer, set to measure absorbance at 340 nm and set the assay temperature (recommended 23-25°C).
2. Zero the spectrophotometer at 340 nm using deionized water.
3. Immediately prior to assay, dilute the sample into Assay Buffer, typically 1/10 (e.g., 30  $\mu$ L + 270  $\mu$ L Buffer)
4. Pipette the following into a cuvette:
  - 350  $\mu$ L Assay Buffer
  - 350  $\mu$ L NADPH Reagent
  - 70  $\mu$ L Sample
5. Place the cuvette in the spectrophotometer.
6. Add to the cuvette: 350  $\mu$ L of dilute tert-Butyl Hydroperoxide. Mix by pipetting up and down twice. Avoid bubbles by keeping tip below the liquid surface.
7. Record the change in  $A_{340}$  for three minutes

Note: The first 15 seconds of the reaction (after adding substrate) should be excluded from data analysis as the rates may not be representative of the enzyme activity due to sample mixing.

Note: It is recommended to run blanks in which 1) the sample is replaced with water, and 2) the sample is assayed with water instead of tert-Butyl Hydroperoxide.

### **Calculations**

1. Determine the rate of decrease in  $A_{340}$  per minute.
2. Calculate the net rate for the sample by subtracting the rate observed for a water blank (water instead of sample).
3. Convert the net  $A_{340}/\text{min}$  for the sample to NADPH consumed (nmol/min/mL) using the following relationship:

$$1 \text{ mU/mL} = 1 \text{ nmol NADPH/min/mL} = (A_{340}/\text{min})/0.00622$$

4. Correct for the dilution of the sample:
  - a. 16 fold dilution in the assay (70  $\mu$ L to 1120  $\mu$ L ).
  - b. Dilution of sample prior to adding to the cuvette.
5. Express the units of activity in the original sample per mL or in relationship to the protein or hemoglobin content (for erythrocytes).

### Example

Calculation of  $A_{340}/\text{min}$  using the Bioxytech® Cellular Glutathione Peroxidase Control assayed at 23°C and a final concentration of 18 mU/mL.

**Table 2: Cellular Glutathione Peroxidase Control**

Time (Seconds)	Absorbance 340 nm	30 Second Interval	$\Delta A_{340}/30 \text{ sec}$
0	1.057		
30	1.003	0 – 30 sec	data not used
60	0.947	30 – 60 sec	0.056
90	0.893	60 – 90 sec	0.054
120	0.836	90 – 120 sec	0.057
150	0.780	120 – 150 sec	0.056
180	0.725	150 – 180 sec	0.055

Average  $\Delta A_{340}/30 \text{ sec}$  0.056  
Sample Rate,  $A_{340}/\text{min}$  0.112  
Blank Rate,  $A_{340}/\text{min}$  (data not shown) 0.003  
Net Rate,  $A_{340}/\text{min}$  0.109

$$1 \text{ mU/mL} = 1 \text{ nmol NADPH/mL/min} = (A_{340}/\text{min})/0.00622$$

$$1 \text{ mU/mL} = 0.109/0.00622 = 17.5 \text{ mU/mL}$$

Correction for sample dilution assay in stock sample:

$$16 \times 17.5 \text{ mU/mL} = 280 \text{ mU/mL}$$

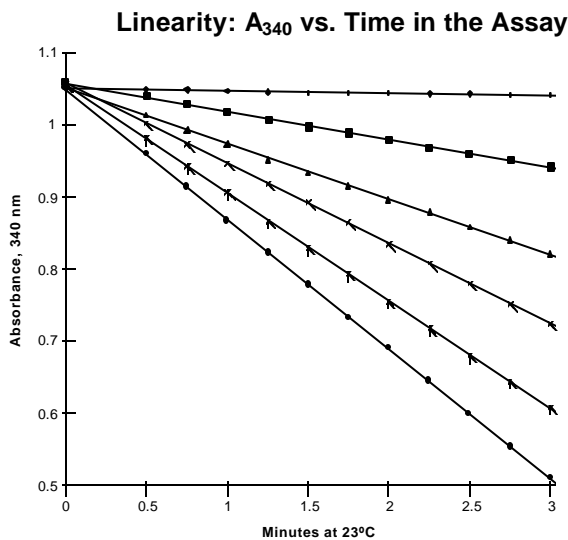
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## PERFORMANCE CHARACTERISTICS

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### Linearity

Shown below is  $A_{340}$  vs. time for a sample of OXIS bovine erythrocyte Glutathione Peroxidase Control, which was assayed at a final concentration of 0, 6, 12, 18, 24, and 30 mU/mL at 23°C (from top to bottom in Figure 1). The absorbance at 340 nm was recorded every 15 seconds. The data points were plotted and regression analysis was used to assign a slope (rate= $A_{340}/\text{min}$ ) to each set of points.



**Figure 1: Linearity: Measured absorbance at 340 nm vs. Time**

In figure 2, the  $A_{340}/\text{min}$  rates (see Figure 1) were corrected for the blank rate and converted into nmol NADPH/min/mL (Y axis). These values were plotted against the mU of bovine erythrocyte GPx added per mL of the reaction mixture. (X axis).

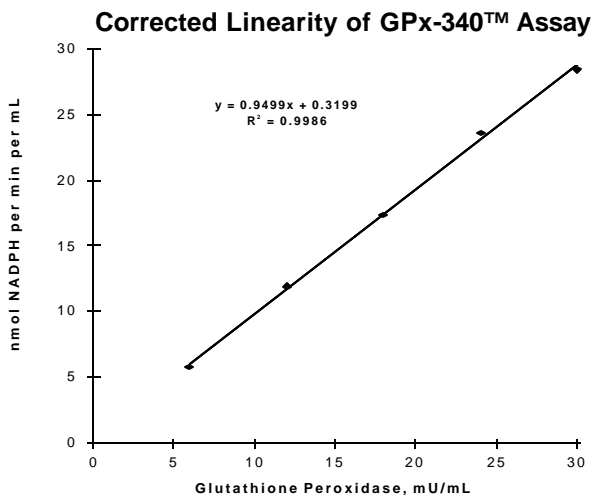


Figure 2. Linearity of GPx-340™ Assay.

### **Total Precision Estimate**

Bovine erythrocyte GPx was assayed 2-3 times a day on 8 different working days at 23 °C to give 20 data sets. These were used to estimate the total precision of the assay, where N = 20.

Table 3: Precision Estimate

mU/mL Added	Average	Standard Deviation	Coefficient of Variation (%)
6.2	6.26	0.25	4.0
18.7	18.85	0.78	4.1
31.25	31.03	1.52	4.9

### **Assay Range**

While samples with a change in absorbance of less than 0.035  $A_{340}/\text{min}$  can be accurately assayed if the protocol is modified (longer reaction times), OXIS recommends that the researcher obtain values from 0.035 to 0.15  $A_{340}/\text{minute}$  using the stated protocol. This corresponds to approximately 5.6 to 24 mU/mL enzyme activity.

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## **NOTES**

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### **Interferences**

- NADPH: enzymes in tissue extracts that consume NADPH can cause a falsely elevated reading in the assay. A blank containing no tert-Butyl Hydroperoxide should be performed to assess nonspecific oxidation of NADPH.
- High concentrations of reducing agents such as dithiothreitol or mercaptoethanol (0.1 mM or greater final concentration in the assay).
- It is reported that falsely elevated values of GPx activity will be observed when erythrocyte lysates are assayed due to heme peroxidase activity by hemoglobin.<sup>7</sup> OXIS studies indicate that there is a negligible contribution to the rate due to hemoglobin in the GPx-340™ Assay. Therefore, it is not recommended to use Drabkin's reagent (potassium ferricyanide/potassium cyanide) to convert hemoglobin to cyanmethemoglobin in the sample as this can cause inactivation of GPx.

- Bovine liver Glutathione S-Transferase was tested for interference in the GPx-340™ Assay. Addition of 17 mU/mL, where 1 unit conjugates 1 μmol of 1-chloro-2,4,-dinitrobenzene with reduced glutathione per minute at pH 6.5, 25 °C, had no effect either on the blank value in the GPx-340™ Assay (no GPx added) or on a sample containing 30 mU/mL bovine erythrocyte c-GPx.
- The use of polyethoxy-nonionic detergents ( e.g., tween, Triton X-100) is not recommended, as they often contain peroxides.

### **Sample Preparation**

- It is advisable to homogenize cells or tissues in a buffer containing a freshly added reducing agent to maintain GPx enzyme activity. For homogenization buffers, it is recommended that 2-mercaptoethanol or dithiothreitol be added at a final concentration of 1 mM. Buffers should be freshly made and used the same day. If homogenates will not be assayed immediately, they should be stored at -70 °C.
- There is abundant c-GPx in erythrocytes. Red blood cells should be washed from tissue samples by perfusion with isotonic saline prior to homogenization. Prior to dissection, animal tissues (brain, kidney, liver, etc.) should be perfused through the heart with 0.9% NaCl containing 0.16 mg/mL heparin.
- Do not use proteolytic enzymes to remove cells from tissue culture plastic. Remove adherent cells from the dish, plate or flask with a rubber policeman.
- General Sample Preparation Protocol:
  1. Homogenize the sample in 4-8 volumes (per weight tissue) of cold buffer e.g.:50 mM TRIS-HCl, pH 7.5, containing 5 mM EDTA and 1 mM 2-mercaptoethanol.
  2. Centrifuge (e.g., 5000-10,000 X g) for 10-20 minutes at 2-8 °C.
  3. Remove the supernatant fluid containing the enzyme.
  4. Freeze samples at -70°C before use, or store on ice if they will be assayed the same day.
  5. Determine the protein concentration of the clarified homogenate to determine the volume to add to the GPx-340™ Assay.
- Sample Preparation for Erythrocyte (RBC) Lysates
  1. Blood should be collected using an anticoagulant such as heparin, citrate or EDTA.
  2. The RBC's should be collected by centrifugation e.g.: 1500 x g for 10 minutes at 4°C.
  3. Draw off the plasma and buffy coat.
  4. Wash the cells once with 10 volumes of cold saline.
  5. Lyse the RBC's by adding 4 volumes of cold deionized water to the packed cells.
  6. Store on ice if they will be assayed the same day, otherwise, freeze at -70°C.
  7. Determine hemoglobin concentration of lysate to determine the appropriate dilution (see above).
- Using the BIOXYTECH® GPx 340™ Assay on a Microtiter Plate Reader:  
If a microtiter plate reader capable of measuring  $A_{340}$  is available, the following may be helpful: The molar extinction coefficient,  $\epsilon$ , for NADPH at 340 nm is  $6220 \text{ M}^{-1} \text{ cm}^{-1}$ . However, the effective path length in microtiter plate readers may not be 1 cm, therefore, the rate of change in the concentration of NADPH cannot be determined using  $\epsilon$ . The researcher must determine a correction factor for the path length in the microtiter plate reader for a specific microtiter plate. A comparison of the rates obtained in a spectrophotometer with a 1 cm path length with those obtained in a microtiter plate using the same samples can be used to determine a correction factor for the path length. The following volumes of reagents and samples would be equivalent to those described for the spectrophotometer:

75 μL Assay Buffer  
 75 μL NADPH Reagent  
 15 μL Sample  
 75 μL 0.007% tert-Butyl Hydroperoxide

### **Limitations**

1. The rate of consumption of NADPH is directly proportional to the GPx concentration over a range from 0.035 A<sub>340</sub>/min up to approximately 0.15 A<sub>340</sub>/min.
  - a. Samples yielding a rate of change greater than 0.15 A<sub>340</sub>/min should be further diluted in Assay Buffer and measured again.
  - b. Samples giving a rate of change of less than 0.035 A<sub>340</sub>/min should be assayed again using smaller dilution (e.g., dilute the stock sample 1/3 or 1/5 prior to assay, instead of 1/10).
2. The temperature of the reaction mixture must be the same each time to avoid variation in the results.
3. The A<sub>340</sub> of the NADPH Reagent will slowly decrease with time after reconstitution. If the A<sub>340</sub> of a complete assay mixture at t = 0 is 0.8, the NADPH Reagent vial in use should be discarded and another vial reconstituted.

### **Assay Performance**

To ensure reproducibility between assays, a glutathione peroxidase control should be used. This control should be frozen in small volumes, preferably at -70 °C, in an appropriate buffer (e.g.: 50 mM Tris-HCl, pH 7.2 containing 5 mM EDTA, 1 mg/mL bovine IgG and 1 mM dithiothreitol or other thiol) at a concentration of at least 3000 mU/mL. OXIS has available a Bioxytech® Cellular Glutathione Peroxidase Control, catalog # 27617Q.

### **Additional Recommendations**

Samples that may be assayed using the GPx-340™ Assay include:

- Purified c-GPx: Purified enzyme can be assayed without any special preparation. However, the enzyme stock should be frozen, preferably at -70 °C at 3000 mU/mL or more, in a buffer containing 1 mM dithiothreitol or 2-mercaptoethanol (or other thiol reducing agent), 5 mM EDTA, and 1 mg/mL protein (for example, bovine IgG) to preserve enzyme activity.
- Erythrocyte lysates: Recommended sample size is 0.2-0.5 mg of hemoglobin or protein (from a clarified lysate) (e.g., 70 µL of 7 mg/mL) added to the reaction mixture.
- Plasma or Serum: Plasma or serum are not recommended for use as samples. It has been demonstrated recently that serum albumin may have peroxidase activity.<sup>8</sup> If the researcher is interested in measuring the secreted (plasma) form of GPx (pl- GPx), OXIS' Bioxytech pl- GPx™ ELISA is recommended.
- Cell and Tissue Homogenates: GPx activity may be assayed in most tissue or cell extracts. It is recommended that approximately 0.1 mg to 1 mg of protein (e.g., 70 µL of 1.5 - 15 mg/mL) be added to the GPx-340 Assay in preliminary experiments to establish the range of activity expected in the particular tissue.

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