OXFORD BIOMEDICAL RESEARCH
P.O. Box 522, Oxford MI 48371 • USA
USA: 800-692-4633 • Fax: 248-852-4466
www.oxfordbiomed.com

Colorimetric Assay for
Cellular Glutathione Peroxidase
Product No. FR 17

For Research Use Only

INTRODUCTION

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.[1] 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_2O_2 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:

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPx} R\text{-}O\text{-}H + GSSG + H_2O \qquad \text{Equation 1}$$

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.[1] 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

This assay is an indirect measure of the activity of c-GPx.[7] 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):

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{c\text{-}GPX} R\text{-}O\text{-}H + GSSG + H_2O \qquad \text{Equation 2}$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \qquad \text{Equation 3}$$

The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 M^{-1} cm^{-1} 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_{340} is recorded. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

REAGENTS

Materials Provided (for 100 tests)

- NADPH Reagent — ß-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
- Cellular Glutathione Peroxidase Control

Warnings and Precautions

Use established laboratory precautions when handling or disposing any chemical contained in this product.

Reagent Storage and Handling

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

PROCEDURE

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 ºC) 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 μL + 270 μL Buffer)
4. Pipette the following into a cuvette:
   - 350 μL Assay Buffer
   - 350 μL NADPH Reagent
   - 70 μL Sample
5. Place the cuvette in the spectrophotometer.
6. Add to the cuvette: 350 μL 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}/min for the sample to NADPH consumed (nmol/min/mL) using the following relationship:

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

4. Correct for the dilution of the sample:
   a. 16 fold dilution in the assay (70 μL to 1120 μL).
   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}/min using a 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	ΔA_{340}/30 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 ΔA_{340}/30 sec	0.056
Sample Rate, A_{340}/min	0.112
Blank Rate, A_{340}/min (data not shown)	0.003
Net Rate, A_{340}/min	0.109

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

1 mU/mL = 0.109/0.00622 = 17.5 mU/mL

Correction for sample dilution assay in stock sample:

16 x 17.5 mU/mL = 280 mU/mL

PERFORMANCE CHARACTERISTICS

Linearity

Shown below is a graph, A_{340} vs. time, for a 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=A340/min) to each set of points.

Figure 1 Linearity: A_{340} vs. Time in the Assay

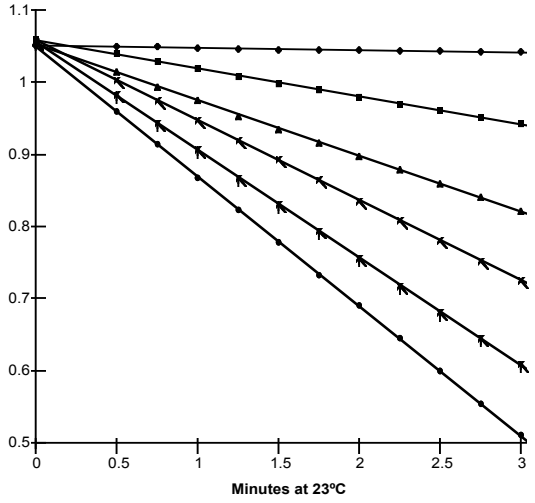
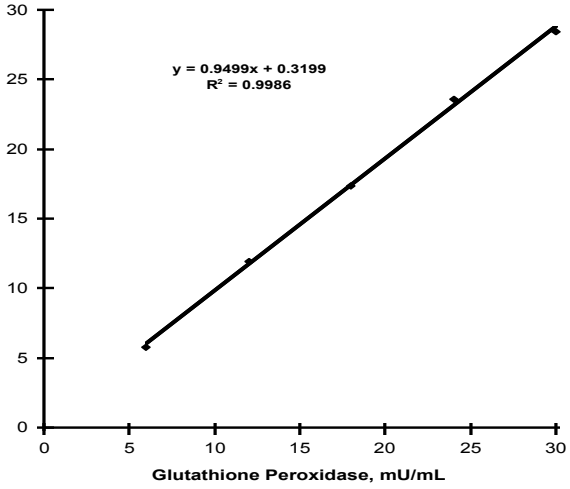


Figure 2 Corrected Linearity of the Assay



In figure 2, the A_{340}/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).

Total Precision Estimate

Bovine erythrocyte GPx was assayed 2-3 times a day on 8 different working days at 23 ºC 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 A340/min can be accurately assayed if the protocol is modified (longer reaction times), it is recommended that the researcher obtain values from 0.035 to 0.15 A_{340}/minute using the stated protocol. This corresponds to approximately 5.6 to 24 mU/mL enzyme activity.

NOTES

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.[7] It has been indicated that there is a negligible contribution to the rate due to hemoglobin in this 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 this 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 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 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 c-GPx 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, ε, for NADPH at 340 nm is 6220 M^{-1} 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 ε. 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 A340/min up to approximately 0.15 A_{340}/min.
   a. Samples yielding a rate of change greater than 0.15 A_{340}/min should be further diluted in Assay Buffer and measured again.
   b. Samples giving a rate of change of less than 0.035 A_{340}/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_{340} of the NADPH Reagent will slowly decrease with time after reconstitution. If the A_{340} 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 ºC, 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.

Additional Recommendations

Samples that may be assessed using this assay include:

- Purified c-GPx: Purified enzyme can be assayed without any special preparation. However, the enzyme stock should be frozen, preferably at -70 ºC 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.[8] If the researcher is interested in measuring the secreted (plasma) form of GPx (pl·GPx), then Oxford Product # FR 16 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 assay in preliminary experiments to establish the range of activity expected in the particular tissue.

REFERENCES

1. F. Ursini, M. Maiorino, R. Brigelius-Flohé, K.D. Aumann, A. Roveri, D. Schomburg, and L. Flohé (1995) Meth. in Enzymol. **252**, 38-53.
2. F.F. Chu (1994) Cytogenet. Cell Genet. **66**, 96-98.

3. G.C. Mills (1957) J. Biol. Chem. **229** 189-197.
4. F.F. Chu, J.H. Doroshow, and S. Esworthy (1993) J. Biol. Chem. **268**, 2571-2576.
5. K.T. Takahashi, N. Avissar, J. Within, and H. Cohen (1987) Arch. Biochm. Biophys. **256**, 677-686.
6. F. Ursini, M. Maiorino, M. Valente, L. Ferri, and C. Gregolin (1982) Biochim. Biophys. Acta **710**, 197-211.
7. D.E. Paglia, and W.N. Valentine (1967) J. Lab. Clin. Med. **70**, 158-169.
8. M.K. Cha, and I.H. Kim (1996) Biochem. Biophys. Res. Commun. **222**, 619-625.

Oxford Biomedical Research, Inc.
P. O. Box 522
Oxford, MI 48371 U.S.A.

Orders: 800-692-4633
Technical Service: 248-852-8815
Fax: 248-852-4466
E-mail: info@oxfordbiomed.com

Last revision April 2001

Made in the U.S.A.