

Glutathione Peroxidase Assay Kit

Catalog No. 703102

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GENERAL INFORMATION

Materials Supplied

Catalog Number	Item	96 Well Quantity/Size	480 Well Quantity/Size
703110	GPx Assay Buffer (10X)	1 vial/3 ml	2 vials/3 ml
703112	GPx Sample Buffer (10X)	1 vial/3 ml	1 vial/3 ml
703114	Glutathione Peroxidase (control)	1 vial/50 µl	1 vial/50 µl
703116	GPx Co-Substrate Mixture	3 vials/40 well size	5 vial/96 well size
703118	GPx Cumene Hydroperoxide	1 vial/2.5 ml	1 vial/12 ml
400014	96-Well Plate	1 plate	5 plates
400012	96-Well Cover Sheets	1 cover	5 covers

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.



WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 340 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

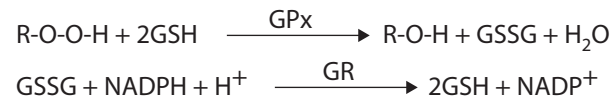
INTRODUCTION

Background

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. With the exception of phospholipid-hydroperoxidase GPx, a monomer, all of the GPx enzymes are tetramers of four identical subunits.^{1,2} Each subunit contains a selenocysteine in the active site which participates directly in the two-electron reduction of the peroxide substrate.^{1,2} The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine.^{1,2}

About This Assay

Cayman's GPx Assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH:



The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in the A₃₄₀ is directly proportional to the GPx activity in the sample.³ The Cayman GPx Assay Kit can be used to measure all of the glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

Reagent Preparation

1. GPx Assay Buffer (10X) - (Catalog No. 703110)

Each vial contains 3 ml of Assay Buffer. Dilute the contents of the vial with 27 ml of HPLC-grade water. This final Assay Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA) should be used in the assay. When stored at 4°C, this diluted Assay Buffer is stable for at least six months. Prepare the additional vial as needed.

2. GPx Sample Buffer (10X) - (Catalog No. 703112)

Dilute 2 ml of Sample Buffer concentrate with 18 ml of HPLC-grade water. This final Sample Buffer (50 mM Tris-HCl, pH 7.6, containing 5 mM EDTA and 1 mg/ml BSA) should be used to dilute the GPx control and the GPx samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least one month.

3. Glutathione Peroxidase (Control) - (Catalog No. 703114)

This vial contains 50 µl of bovine erythrocyte GPx. To avoid repeated freezing and thawing, the GPx should be aliquoted into several small vials and stored at -20°C. Prior to use, transfer 10 µl of the supplied enzyme to another vial and dilute with 490 µl of diluted Sample Buffer and keep on ice. The diluted enzyme is stable for four hours on ice. A 20 µl aliquot of this diluted enzyme per well causes a decrease of approximately 0.051 absorbance unit/minute under the standard assay conditions described in Performing the Assay (see page 11).

4. GPx Co-Substrate Mixture - (Catalog No. 703116)

The 96-well kit contains three 40 well size of Co-Substrate Mixture. These vials contain a lyophilized powder of NADPH, glutathione, and glutathione reductase. Each reconstituted vial will be enough reagent for 40 wells. Reconstitute the number of vials that you will need by adding 2 ml of HPLC-grade water to each vial and vortex well. The 480-well kit contains five 96 well size of Co-Substrate Mixture. Reconstitute the number of vials that you will need by adding 6 ml of HPLC-grade water to each vial and vortex well. Each reconstituted vial will be enough reagent for 96 wells. The reconstituted reagent should be kept at 25°C while assaying and then stored at 4°C. If stored at 4°C, the reconstituted reagent is stable for two days. *NOTE: Do not freeze the reconstituted reagent.*

5. GPx Cumene Hydroperoxide - (Catalog No. 703118)

The 96-well kit contains one 2.5 ml vial of cumene hydroperoxide. The 480-well kit contains one 12 ml vial of cumene hydroperoxide. The vials should be stored at -20°C when not being used. The reagent is ready to use as supplied.

Sample Preparation

Tissue Homogenate

1. Prior to dissection, perfuse or rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes, rather use a rubber policeman.
2. Homogenize cell pellet in cold buffer (*i.e.*, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma and Erythrocyte Lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipet off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in 4 volumes of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) for assaying and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

NOTE: It has been reported that heme peroxidase activity of hemoglobin can lead to falsely elevated GPx activity in erythrocyte lysates. There was no significant effect in the GPx activity when assayed with cumene hydroperoxide as the substrate. Therefore, it is not necessary to treat the sample with Drabkin's Reagent (potassium ferricyanide/potassium cyanide) to convert hemoglobin to cyanmethemoglobin before assaying.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as non-enzymatic or background wells. The absorbance rate of these wells must be subtracted from the absorbance rate measured in the GPx sample and control wells. We suggest that there be at least three wells designated as positive controls and that you record the contents of each well on the template sheet provided on page 19.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	7	7	7	15	15	15	23	23	23
B	C	C	C	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

B - Background Wells
C - Positive Control Wells
1-30 - Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Use different tips to pipette the assay buffer (dilute), co-substrate mixture, enzymes, and cumene hydroperoxide.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 190 μl in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- Use the Assay Buffer (dilute) in the assay.
- Monitor the decrease in absorbance at 340 nm using a plate reader.

Performing the Assay

1. **Background or Non-enzymatic Wells** - add 120 μl of Assay Buffer and 50 μl of co-substrate mixture to three wells.
2. **Positive Control Wells (bovine erythrocyte GPx)** - add 100 μl of Assay Buffer, 50 μl of co-substrate mixture, and 20 μl of diluted GPx (control) to three wells.
3. **Sample Wells** - add 100 μl of Assay Buffer, 50 μl of co-substrate mixture, and 20 μl of sample to three wells. To obtain reproducible results, the amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with Sample Buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 10,000 to bring the enzymatic activity to this level. *NOTE: The amount of sample added to the well should always be 20 μl . To determine if an additional sample control should be performed see the Interferences section (page 14).*
4. Initiate the reactions by adding 20 μl of cumene hydroperoxide to all the wells being used. Make sure to note the precise time the reaction is initiated and add the cumene hydroperoxide as quickly as possible.
5. Carefully shake the plate for a few seconds to mix.
6. Read the absorbance once every minute at 340 nm using a plate reader to obtain at least 5 time points. *NOTE: The initial absorbance of the sample wells should not be above 1.2 or below 0.5.*

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute by:
 - a. Plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown on page 13 using bovine erythrocyte GPx) -or-
 - b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{340}/\text{min.} = \frac{A_{340}(\text{Time 2}) - A_{340}(\text{Time 1})}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

2. Determine the rate of $\Delta A_{340}/\text{min.}$ for the background or non-enzymatic wells and subtract this rate from that of the sample wells.
3. Use the following formula to calculate the GPx activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of $0.00373 \mu\text{M}^{-1}$ *. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP^+ per minute at 25°C .

$$\text{GPx activity} = \frac{\Delta A_{340}/\text{min.}}{0.00373 \text{ M}^{-1}} \times \frac{0.19 \text{ ml}}{0.02 \text{ ml}} \times \text{Sample dilution} = \text{nmol/min/ml}$$

*The actual extinction coefficient for NADPH at 340 nm is $0.00622 \mu\text{M}^{-1}\text{cm}^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.6 cm).

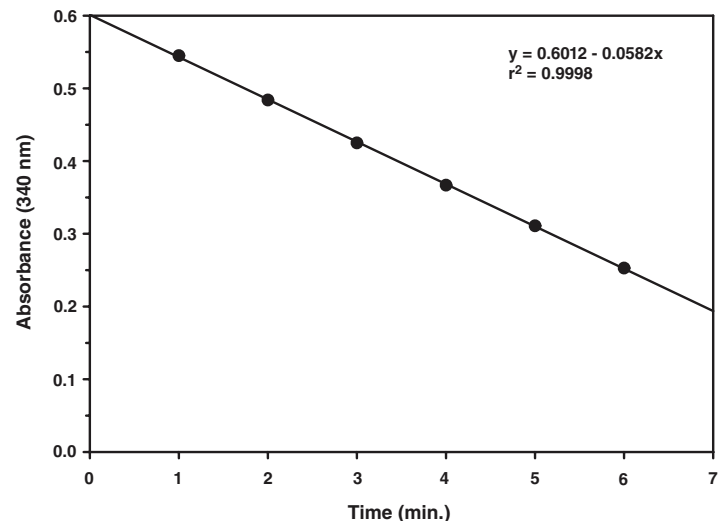


Figure 1. Activity of bovine erythrocyte GPx

Performance Characteristics

Precision:

When a series of seventy-seven GPx measurements were performed on the same day, the intra-assay coefficient of variation was 5.7%. When a series of seventy-seven GPx measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 7.2%.

Assay Range:

The dynamic range of the assay is limited only by the accuracy of the absorbance measurement. Most plate readers are linear to an absorbance of 1.2. Samples containing GPx activity between 50-344 nmol/min/ml can be assayed without further dilution or concentration. This GPx activity is equivalent to an absorbance decrease of 0.02 to 0.135 per minute.

Interferences

- Samples that have a high intrinsic absorbance at 340 nm may exceed the absorbance maximum of the plate reader. Therefore, samples with an initial absorbance >1.2 should be diluted with Sample Buffer until the absorbance is lowered. For example, hemoglobin absorbs significantly at 340 nm, and thus erythrocyte lysates must be diluted before assaying.
- Samples containing high levels of GSSG or NADPH consuming enzymes will cause the GPx levels to be overestimated. A blank without cumene hydroperoxide should be performed to assess non-specific oxidation of NADPH. GSSG can be removed from the sample by either dialysis or passing through a gel filtration column.

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	Phosphate	No
Detergents:	CHAPS ($\leq 1\%$)	No
	Triton X-100 ($\leq 1\%$)	No
	Tween 20 ($\leq 1\%$)	No
Protease Inhibitors/ Chelators:	Antipain (≤ 0.1 mg/ml)	No
	Chymostatin	Yes
	Leupeptin (≤ 10 μ g/ml)	No
	PMSF (≤ 200 μ M)	No
	Trypsin (≤ 10 μ g/ml)	No
	EDTA (≤ 5 mM)	No
	EGTA (≤ 5 mM)	No
Solvents:	Ethanol (10 μ l)	No
	Methanol (10 μ l)	No
	Dimethylsulfoxide (10 μ l)	No
Others:	Bovine serum albumin ($\leq 1\%$)	No
	Glycerol ($\leq 10\%$)	No
	2-Mercaptoethanol	Yes

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Carefully tap the side of the plate with your finger to remove bubbles B. Be careful not to splash the contents of the wells
The initial absorbance in the wells is less than 0.1	Co-substrate mixture was not added to the wells	Make sure to add all components to the wells
No decrease in absorbance was observed in the sample wells	A. Enzyme activity was too low B. Cumene Hydroperoxide was not added to the wells	A. Concentrate your sample using an Amicon centrifuge concentrator with a 10,000 MW cut-off and re-assay. Make sure to add all components to the wells
Reaction rate was too fast. The initial absorbance of the sample well is below 0.5	Too much enzyme added to well(s)	Dilute your samples with diluted sample buffer and re-assay
The initial absorbance in the sample wells is above 1.2		Dilute your sample with diluted sample buffer and re-assay

Additional Reading

Go to www.caymanchem.com/703102/references for a list of publications citing the use of Cayman's Glutathione Peroxidase Assay Kit.

References

1. Ursini, F., Maiorino, M., and Gregolin, C. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochim. Biophys. Acta* **839**, 62-70 (1985).
2. Forstrom, J.W., Zakowski, J.J., and Tappel, A.L. Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry* **17**, 2639-2644 (1978).
3. Paglia, D.E. and Valentine, W.N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158-169 (1967).

Related Products

Aconitase Assay Kit - Cat. No. 705502
Antioxidant Assay Kit - Cat. No. 709001
Catalase Assay Kit - Cat. No. 707002
Glutathione Assay Kit - Cat. No. 703002
Glutathione Reductase Assay Kit - Cat. No. 703202
Glutathione S-Transferase Assay Kit - Cat. No. 703302
Hydrogen Peroxide (urinary) Assay Kit - Cat. No. 706011
4-hydroxy Hexenal - Cat. No. 32060
4-hydroxy Nonenal - Cat. No. 32100
8-Isoprostane EIA Kit - Cat. No. 516351
Lipid Hydroperoxide Assay Kit - Cat. No. 705002
Protein Carbonyl Assay Kit - Cat. No. 10005020
Superoxide Dismutase Assay Kit - Cat. No. 706002
TBARS Assay Kit - Cat. No. 10009055
Thioredoxin Reductase Assay Kit - Cat. No. 10007892
Xanthine Oxidase Assay Kit - Cat. No. 10010895

NOTES

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