Glutathione Peroxidase Assay Kit

Item No. 703102

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	96 Well Quantity/Size	480 Well Quantity/ Size	Storage
703110	GPX Assay Buffer (10X)	1 vial/5 ml	2 vials/5 ml	-20°C
703112	GPX Sample Buffer (10X)	1 vial/3 ml	1 vial/3 ml	-20°C
703114	Glutathione Peroxidase (control)	1 vial/50 µl	1 vial/50 µl	-20°C
703111	GPX Co-Substrate Mixture	2 vials	5 vials	-20°C
703118	GPX Cumene Hydroperoxide	1 vial/2.5 ml	1 vial/12 ml	-20°C
703119	GPX NADPH	2 vials	5 vials	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	5 plates	RT
400012	96-Well Cover Sheet	1 cover	5 covers	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3640

Email: techserv@caymanchem.com

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 in the Materials Supplied section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance at 340 nm
2. Adjustable pipettes and a multichannel pipette
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-hydroperoxide 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:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPX}} \text{R-O-H} + \text{GSSG} + \text{H}_2\text{O}$$

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

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 A_{340} 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.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **GPX Assay Buffer (10X) - (Item No. 703110)**

   This vial contains 5 ml of a 10X Assay Buffer solution. Once thawed, mix 5 ml GPX Assay Buffer (10X) with 45 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) - (Item No. 703112)**

   This vial contains 3 ml of a 10X Sample Buffer solution. Once thawed, mix 2 ml GPX Sample Buffer (10X) 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) is used to dilute the GPX control (Item No. 703114) and the samples prior to assaying. When stored at 4°C, this diluted Sample Buffer is stable for at least one month.

3. **Glutathione Peroxidase (Control) - (Item No. 703114)**

   This vial contains 50 µl of bovine erythrocyte GPX. To avoid repeated freeze/thaw, 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 on ice, and add 490 µl of diluted Sample Buffer. The diluted enzyme is stable for four hours on ice. *NOTE: A 20 µl aliquot of this diluted enzyme per well results in a decrease of approximately 0.051 absorbance unit/minute under the standard assay conditions described in* ***Performing the Assay*** *(see page 14).*

4. **GPX Co-Substrate Mixture - (Item No. 703111)**

   This vial contains lyophilized glutathione and glutathione reductase. Reconstitute the contents of the vial with 6 ml of diluted GPX Assay Buffer. One vial of GPX Co-Substrate is sufficient reagent to assay 96 wells. Reconstitute additional vials as needed. The reconstituted GPX Co-Substrate is stable at room temperature for two hours or for two days when stored at 4°C. ***NOTE: Do not freeze the reconstituted reagent.***

5. **GPX NADPH - (Item No. 703119)**

   This vial contains lyophilized NADPH. Reconstitute the vial with 6 ml of diluted GPX Assay Buffer. One vial of GPX NADPH is sufficient reagent to assay 96 wells. Reconstitute additional vials as needed. The reconstituted NADPH is stable at room temperature for two hours or for two days when stored at 4°C.

6. **GPX Cumene Hydroperoxide - (Item 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 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 the 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. Pipette 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. Dilute the plasma 1:2 with Sample Buffer before assaying.
4. Remove the white buffy layer (leukocytes) and discard.
5. Lyse the erythrocytes (red blood cells) in 4 volumes of ice-cold HPLC-grade water.
6. Centrifuge at 10,000 x g for 15 minutes at 4°C.
7. 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.
8. Dilute the erythrocyte lysate 1:10-1:20 with Sample Buffer before assaying.

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.

Tissue Homogenization using the Precellys 24 Homogenizer

- Freeze organs immediately upon collection and then store at -80°C. Snap-freezing of tissues in liquid nitrogen is preferred.
- Add 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA and 1 mM DTT) per 100 milligrams of tissue.
- Homogenize the sample using the Precellys 24 according to appropriate settings:

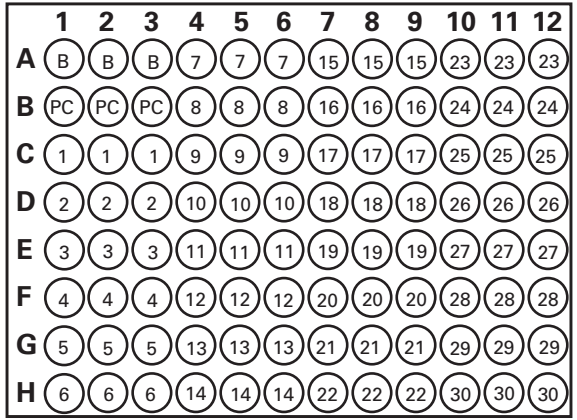
Organ	Speed (rpm)	Cycle Length (seconds)	Cycle Break (seconds)	Number of Cycles	Beads
Heart (aorta)	5,000	30	30	3	CK28 Large Ceramic

- Spin the tissue homogenates at 10,000 x g for 15 minutes at 4°C.
- Collect supernatant and assay samples according to the kit booklet protocol. Samples may need to be diluted appropriately for assay and should be normalized using a protein assay.

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 are at least three wells designated as positive controls and that you record the contents of each well on the template sheet provided on page 22.



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

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells.
- Use different tips to pipette the diluted Assay Buffer, Co-Substrate, NADPH, 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 is performed at room temperature.
- Monitor the decrease in absorbance at 340 nm.

Performing the Assay

1. **Background Wells** - add 70 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 50 µl NADPH to three wells.
2. **Positive Control Wells** - add 50 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, 50 µl NADPH, and 20 µl of diluted GPX (control) to three wells.
3. **Sample Wells** - add 50 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, 50 µl NADPH, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GPX added to the well should decrease the absorbance to 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 18).*
4. Initiate the reactions by quickly adding 20 µl of Cumene Hydroperoxide to all the wells being used.
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.*

ANALYSIS

Calculations

1. Determine the change in absorbance (ΔA_{340}) per minute by:
   a. Plot 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 16 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:

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

*Use the absolute value.

2. Determine the rate of ΔA_{340}/min. for the background of 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 μM^{-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.

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

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

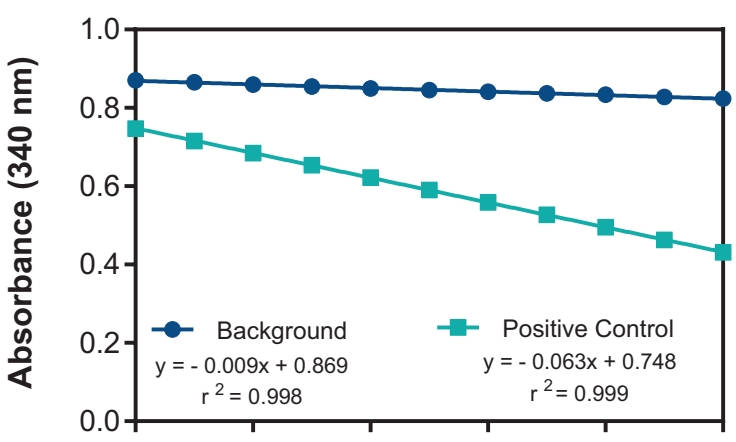


Figure 1. Glutathione Peroxidase (control) Activity

Performance Characteristics

Precision:

When a series of 77 GPX measurements were performed on the same day, the intra-assay coefficient of variation was 5.7%. When a series of 77 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 (≤ 1%)	No
	Triton X-100 (≤ 1%)	No
	Polysorbate 20 (≤ 1%)	No
Protease Inhibitors/ Chelators:	Antipain (≤0.1 mg/ml)	No
	Chymostatin	Yes
	Leupeptin (≤10 µg/ml)	No
	PMSF (≤200 µM)	No
	Trypsin (≤10 µg/ml)	No
	EDTA (≤5 mM)	No
	EGTA (≤5 mM)	No
Solvents:	Ethanol (10 µl)	No
	Methanol (10 µl)	No
	Dimethylsulfoxide (10 µl)	No
Others:	Bovine serum albumin (≤ 1%)	No
	Glycerol (≤ 10%)	No
	2-Mercaptoethanol	Yes

Troubleshooting

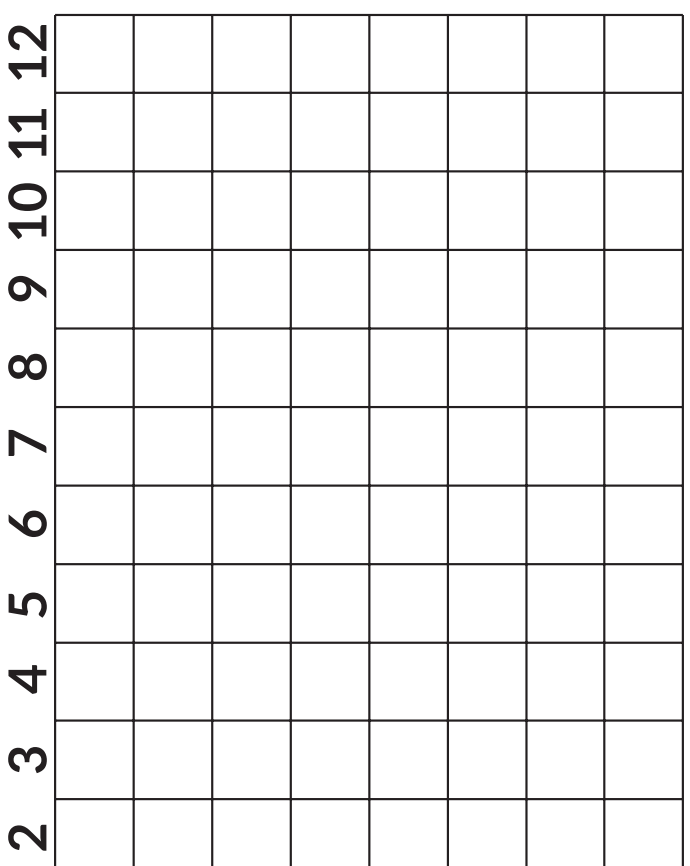
Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
The initial absorbance 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).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.