User Manual

Glutathione Peroxidase Assay Kit

353919

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Not for use in diagnostic procedures. Not for human or animal consumption.

Product Overview

Glutathione peroxidase (glutathione peroxidase) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. Apart from phospholipid-hydroperoxide Glutathione peroxidase, a monomer, all the glutathione peroxidase enzymes are tetramers of four identical subunits.[1,2] Each subunit contains a selenocysteine in the active site that 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]

Principles of the Assay

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

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

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

The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the glutathione peroxidase activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the glutathione peroxidase activity in the sample.[3] The assay can be used to measure all the glutathione-dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates.

Materials Provided

- 10X Assay Buffer (KP31672-5ML): 1 vial, 5 mL
- 10X Sample Buffer (KP31673-3ML): 1 vial, 3 mL
- Glutathione Peroxidase (Control) (KP31674-50UL): 1 vial, 50 µL
- Co-Substrate Mixture (KP31880-1EA): 2 vials
- Cumene Hydroperoxide (KP31676-2.5ML): 1 vial, 2.5 mL
- NADPH (KP31881-1EA): 2 vials
- 96-Well Plate and (KP31625-1EA): 1 plate, 1 plate sealer

Materials Required (Not supplied)

- Plate reader with a 340 nm filter
- Adjustable pipettors
- Repeat pipettor
- Source of pure water. Glass distilled water or HPLC-grade water is acceptable.
- Phosphate Buffer Saline (PBS)
- Homogenization buffer (cell and tissue preparation)

Warnings and Precautions

Read instructions carefully before beginning this assay.

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver dilute Assay Buffer, co-substrate mixture, and cumene hydroperoxide to the wells. This saves time and helps to maintain more precise incubation times.
- Use different tips to pipet the dilute Assay Buffer, co-substrate mixture, enzymes, and cumene hydroperoxide.
- Before pipetting each reagent, equilibrate the pipet tip in that reagent (slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the well.

Storage and Stability

Store at –20 °C.

Protocol

Sample Preparation

Tissue Homogenate

Prior to dissection, perfuse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, containing 0.16 mg/mL heparin to remove any red blood cells and clots.

1. Homogenize the tissue in 5-10 mL cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram tissue.
2. Centrifuge at 10,000 x *g* for 15 minutes at 4 °C.
3. 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 (1000-2000 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 homogenization buffer (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. 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 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.

   Note: It has been reported that heme peroxidase activity of hemoglobin can lead to falsely elevated Glutathione peroxidase activity in erythrocyte lysates. There was no significant effect in the Glutathione peroxidase 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.

Reagent Preparation

1X Assay Buffer

Dilute 5 mL Assay Buffer concentrate with 45 mL HPLC-grade water. This final diluted 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.

1X Sample Buffer

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

Glutathione Peroxidase (Control)

This vial contains a solution of bovine erythrocyte glutathione peroxidase. To avoid repeated freezing and thawing, the Glutathione Peroxidase (Control) should be dispensed into small aliquots and stored at -20 °C. Prior to use, transfer 10 µL of the Glutathione Peroxidase (Control) to another vial and dilute with 490 µL diluted Sample Buffer and keep on ice. The diluted Glutathione Peroxidase (Control) is stable for 4 hours on ice.

Note: A 20 µL aliquot of this diluted Glutathione Peroxidase (Control) per well causes a decrease of approximately 0.051 absorbance unit/minute under the standard assay conditions described in the Detailed Protocol.

Co-Substrate Mixture

These vials contain a lyophilized powder of glutathione and glutathione reductase. Each reconstituted vial will be enough reagent for 60 wells. Reconstitute the contents of the vial with 3 mL diluted Assay Buffer; reconstitute additional vials as needed. 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 2 days. Do not freeze the reconstituted reagent.

NADPH

Reconstitute the vial with 3 mL diluted Assay Buffer. One vial is sufficient for 60 wells. Reconstitute additional vials as needed. The reconstituted NADPH is stable at room temperature for two hours or for two days at 4 °C.

Cumene Hydroperoxide

This vial contains a solution of cumene hydroperoxide and should be stored at -20 °C when not being used. The reagent is ready to use as supplied.

Technical Hints

- 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 diluted Assay Buffer in the assay.
- Monitor the decrease in absorbance at 340 nm using a plate reader.
- There is no specific pattern for using the wells on the plate. However, it is necessary to have 3 wells designated as non-enzymatic or background wells. The absorbance rate of these wells must be subtracted from the absorbance rate measured in the glutathione peroxidase sample and control wells. We suggest that there be at least 3 wells designated as positive controls.

Detailed Protocol

1. **Background or Non-enzymatic Wells:** Add 70 µL diluted Assay Buffer and 50 µL Co-Substrate Mixture, and 50 µL NADPH to 3 wells.
2. **Positive Control Wells (bovine erythrocyte glutathione peroxidase):** Add 50 µL diluted Assay Buffer, 50 µL of Co-Substrate Mixture, 50 µL NADPH, and 20 µL diluted Glutathione Peroxidase (Control) to 3 wells.
3. **Sample Wells:** Add 50 µL diluted Assay Buffer, 50 µL of Co-Substrate Mixture, 50 µL NADPH, and 20 µL sample to 3 wells. To obtain reproducible results, the amount of glutathione peroxidase added to the well should cause an absorbance decrease between 0.02 and 0.135/minute. When necessary, samples should be diluted with diluted 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.
4. Initiate the reactions by adding 20 µL 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.

Data Analysis

Calculating the Results

Determination of the reaction rate

1. Determine the change in absorbance (ΔA_{340}) per min by:
   - Plotting the absorbance values as a function of time to obtain the slope (rate) of the linear portion of the curve (Refer to Figure 1).

     -or-

   - Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

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

* Use absolute value

2. Determine the rate of ΔA_{340}/minute. 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 Glutathione peroxidase 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.

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

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

Assay Characteristics and Examples

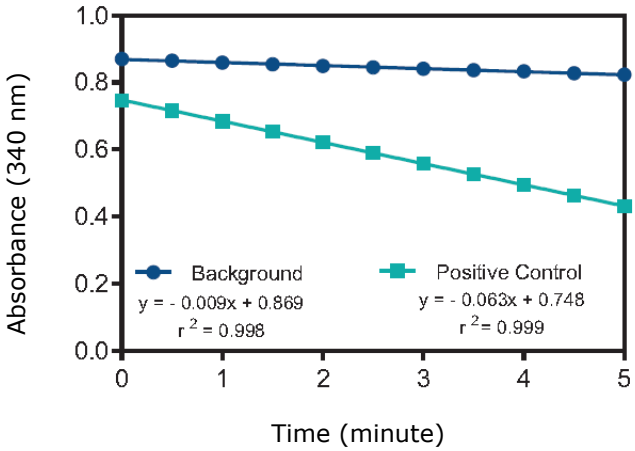

Figure 1: Activity of Bovine Erythrocyte Glutathione Peroxidase

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 glutathione peroxidase activity between 50-344 nmol/min/mL can be assayed without further dilution or concentration. This glutathione peroxidase activity is equivalent to an absorbance decrease of 0.02 to 0.135 per minute.

Results

- 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:

Components	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

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).

Troubleshooting

Problem	Causes	Solutions
Erratic values; dispersion of duplicates/triplicates	Poor pipetting/technique	Be careful not to splash the contents of the wells
	Bubble in the well(s)	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	Enzyme activity was too Low.	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
	Cumene Hydroperoxide was not added 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.

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