ZeptoMetrix®

TOTAL GLUTATHIONE PEROXIDASE ASSAY KIT

Catalog Number:
0805002A

DESCRIPTION AND INTENDED USE

Glutathione peroxidase (GPx, EC# 1.11.1.9) is an enzyme found in cytoplasmic and mitochondrial fractions of cells. GPx acts on lipid hydroperoxide (LHP) substrates that are released from membrane phospholipids by phospholipase A2 [1]. It can utilize cholesterol hydroperoxide [2] and hydrolyzes H_2O_2 at low concentrations. [3] The antioxidant enzyme catalyzes the reduction of hydrogen peroxide and hydroperoxides formed from fatty acids, thereby effectively removing toxic peroxides from living cells. It plays the important role of protecting cells from potential damage by free radicals, formed by peroxide decomposition. [4, 5] The activity of GPx is coupled to glutathione reductase (GSSG-R), which maintains reduced glutathione (GSH) levels [6]. Using glutathione (GSH) as a reducing reagent, the GPx enzymes catalyze the reduction of H_2O_2 and organic peroxides (R-O-O-H) to water and the corresponding stable alcohol thus inhibiting the formation of free radicals. Enzyme activity can be decreased by negative feedback from excess substrate or from damage by oxidative modification.[7]

Oxidative stress has been implicated in aging and in the pathogenesis of a number of disorders. The extent of injury is generally related to an increase or decrease of one or more free radical scavenging enzymes of which GPx is one. [8] Various diseases show different levels of the universally present GPx in all tissues. A reduction in enzyme level is associated with Parkinson's disease [9] and in Chronic Glomerulonephritis patients. [10] In patients with end-stage renal disease (ESRD), GPx activity in adult patients was comparable to that in the control groups (children and adults); the GPx in children with ESRD was almost twice as high than in the other groups. [11] GPx activity decreased significantly in liver and increased in kidney in three-week-old diabetic rats that showed a reversal with a change in diet.[12]

The OXItek "Total Glutathione Peroxidase Assay Kit" provides a method of quantifying the activity of total glutathione peroxidase.

***The Total Glutathione Peroxidase Assay Kit is for Research Purposes Only.**

PRINCIPLE OF THE PROCEDURE

The general reaction performed at 25ºC and pH 8.0, catalyzed by GPx can be described as follows:

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

In this assay, cumene hydroperoxide is used as the peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and NADPH (β–Nicotinamide Adenine Dinucleotide Phosphate, Reduced) are included in the reaction mixture. The formation of GSSG (glutathione) catalyzed by GPx is coupled to the recycling of GSSG back to GSH using GSSG-R. NADPH is oxidized to $NADP^+$. The change in A_{340} due to NADPH oxidation is monitored and is indicative of GPx activity. Since all other reagents are provided in excess, the amount of GPx in the test sample is the rate-limiting factor. The over-all 2-step reaction is: [13]

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + H_2O + \text{GSSG} \xrightarrow[\text{NADPH} \rightarrow \text{NADP}^+]{\text{GSSG-R}} 2\text{GSH}$$

The oxidation of NADPH to $NADP^+$ is monitored spectrophotometrically by a decrease in absorbance at 340 nm (A_{340}). 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. [13]

Cumene Hydroperoxide is used to measure the total GPx activity. This substrate is suitable for the reaction because it has a low spontaneous reaction with GSH, low spontaneous hydrolysis and is not metabolized by Catalase, one of the other universally present antioxidant enzymes.

GPx activity both in plasma and in Red Blood Cells (hemolysate) can be determined with this kit. The kit can also be adapted to GPx activity determination in cells from culture and tissue homogenates.

The Kit provides reagents sufficient for 100 (~1mL) tests.

REF	Catalog Number		Temperature Limitation
LOT	Batch Code		Expiration Date
RUO	For Research Use Only		Manufacturer

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PRECAUTIONS

- Please read all instructions carefully prior to performing assay. Recommendations in the insert are to be used as guidelines.
- To avoid cross contamination, use separate pipette tips for each sample.
- Universal safety precautions while working with bio-hazardous materials should be adopted. (14)
- Wear gloves, lab coats and safety glasses at all times.
- All contaminated materials and biohazardous material should be properly disposed and work surfaces appropriately decontaminated.
- The source for the QC Material is animal based and considered non-infectious. All tests with other samples should be conducted using precautions recommended for blood borne pathogens, as defined by OSHA regulations.

REAGENTS

Materials Supplied:

- **GPx-Assay Buffer:** (120 mL); contains Potassium Phosphate and EDTA.
- **GPx-Reagent 1:** (45 mg); contains reduced Glutathione (GSH).
- **GPx Reagent 2:** (5 mg); contains NADPH.
- **Gpx-Reagent 3:** (10 mL); contains Cumene Hydroperoxide, Sodium Bicarbonate.
- **GPx-Reagent 4:** (1 mL); contains Potassium Phosphate and GSSG-R (Glutathione Reductase).
- **QC Material:** (0.5 mL); contains animal source material.

Handling and Storage:
Store GPx-Reagent 1, GPx-Reagent 2 and GPx-Reagent 4 at ≤ -65ºC. GPx-Reagent 3 and the GPx-Assay Buffer should be stored at 4ºC.The components of the Kit are stable for 1 year when stored properly. The QC material when stored at ≤ -65ºC is stable for 1 year unreconstituted.

Materials/Equipment/Procedures Required But Not Supplied

- UV/Vis spectrophotometer with a kinetic program. Should preferably be equipped with temperature controlled cuvette chamber.
- Spectrophotometric cuvettes.
- Adjustable pipettors with disposable pipette tips.
- Serological pipettes.
- Saline.
- Beakers/flasks to make reagents and working solutions.
- Deionized distilled water (DI).
- Hemoglobin measurement for red blood cell hemolysate.
- Protein measurements for clarified homogenates from tissues.

PREPARATION OF REAGENTS AND EQUIPMENT

- **Working Solution**:
  - Use aseptic handling to aliquot 25 mL of GPx-Assay Buffer to a glass beaker. Add entire contents of 1 vial of GPx-Reagent 1. Wash vial with buffer to ensure complete recovery.
  - Add 200 µl GPx-Reagent 4 to the above solution. Bring to room temperature. Working Solution is good for 25 (1 mL) assays.
  - This solution can be kept at ambient temperature (24-25ºC) for a period of 8-10 hours with no significant effect on assay performance. Do not freeze solution.
- **Start Solution**:
  - Add 2.14 mL GPx-Reagent 3 to 1 vial of GPx-Reagent 2. Bring to room temperature.
  - The start solution can be kept at ambient temperature (24-25ºC) for a period of 8-10 hours with no significant effect on assay performance. Do not freeze solution.
- **QC Material**:
  - Add .5 mL of DI water to the QC Material vial. Allow to sit 5 min, then vortex gently until completely resuspended. Keep on ice.
- Turn on spectrophotometer, set to measure absorbance at 340 nm in the kinetic mode. Lag time is 40 sec, rate time 60 sec, read intervals 15-30 sec. Assay temperature should be set at 24-25ºC.

Notes:
- Samples should be run in duplicate.
- Reactions are very sensitive to temperature changes.
- Blanks (DI water replaces sample) should be run.
- Sodium azide inhibits the reaction.
- Thawed and resuspended QC Material should not be refrozen. Recommend each lab have plasma they can run each time.

SAMPLE PREPARATION

PLASMA

1. Collect blood using EDTA, heparin or citrate as the anticoagulant.
2. Spin down the RBC by centrifugation at 3000 rpm for 10 min at 4ºC
3. Remove plasma from the cells by drawing it off from the top.
4. If not used directly, quick freeze samples in either liquid nitrogen or ethanol-dry ice bath and store at ≤ -65ºC freezer until analysis.
5. Thaw out samples before analysis. Vortex well to mix.

RED BLOOD CELLS

1. Collect and process blood as above till step 3.
2. Remove the buffy coat (the white interface between the pelleted RBCs and the plasma) and discard.
3. Wash RBC pellet with saline at 4ºC. Centrifuge at 3000 rpm for 10 min at 4ºC. Discard clear saline from top. Repeat once.
4. To an aliquot of RBC add an equal volume of DI water, vortex well for complete lysis of the cells.
5. Perform a hemoglobin measurement of this 1:2 diluted RBC hemolysate. Convert from g/dl to g/L.
6. If not used directly, quick freeze hemolysate sample aliquots in either liquid nitrogen or ethanol-dry ice bath and store at ≤ -65ºC freezer until analysis.
7. Thaw out frozen samples before analysis. Vortex well.
8. Dilute thawed out red blood cell lysate in DI water to 6-7 g/L of hemoglobin.
   - Mix well.
   - Leave diluted samples at RT or on ice before analysis.
   - Diluted samples are stable for 8 hours at RT or 48 hours at 0-5ºC.

TISSUES

1. Homogenize tissue samples in 4-6 volumes (per wet weight of tissues) of cold GPx Assay Buffer and 1 mM β-mercaptoethanol.
2. Centrifuge mixture for 10-15 min at 8000 rpm at 4ºC.
3. Remove supernatant from the top of the tube for the assay.
4. Determine protein concentration of the supernatant.
5. If not used directly, quick freeze samples in either liquid nitrogen or ethanol-dry ice bath and store at ≤ -65ºC freezer until analysis.
6. Thaw out samples before use. Vortex well.

YEAST CELLS (Maximum sample: 10^9 cells)

1. Pellet yeast cells (10^9 cells) by centrifugation at 2500 rpm for 10 min in 13 X 100 mm glass test tubes.
2. Resuspend cell pellet in 1.25 mL cold extraction buffer (20 mM Tris pH 8.0).
3. Add 0.48 g glass beads (0.22 mm).
4. Vortex samples for 5 min.
5. Centrifuge samples at 2500 rpm for 10 min.
6. Aliquot supernatants to Eppendorf tubes.
7. If not used directly, quick freeze samples in either liquid nitrogen or ethanol-dry ice bath and store at ≤ -65ºC freezer until analysis.
8. Thaw out samples before use. Vortex well.

ASSAY PROCEDURE

Step 1: Turn on spectrophotometer and allow instrument to initialize for 15 minutes. Set instrument for kinetic measurements at 340 nm and 25ºC.

Step 2: Zero spectrophotometer at 340 nm with DI water.

Step 3: Pipette the following reagents into the cuvette:

- 870 µl Working Solution A
- 60 µl Start Solution

Step 4: Start the reaction by adding 30 µl sample or DI water for blank determinations. Pipette up and down to mix thoroughly. Avoid bubbling.

Step 5: Cover cuvette with parafilm and invert gently 2-3 times.

Step 6: Place cuvette in the correct position in the spectrophotometer.

Step 7: Record the change in A_{340} for 1 min, following a 40 sec lag time.

CALCULATIONS

Step 1: The net rate of decrease in A_{340} for the sample can be calculated by subtracting the rate observed for a blank (where water is used instead of sample) from the rate observed for each sample.

Step 2: The net A_{340}/min for the test sample can be converted to NADPH consumed using the following relationship:

1 unit of Glutathione Peroxidase will cause the formation of 1 μmol $NADP^+$ from NADPH per min at pH 8.0 at 25ºC.

Extinction coefficient for NADPH is 0.00622 μM^{-1} cm^{-1} at 340 nm.

Step 3: Activity of GPx can be expressed as International Unit/Liter (U/L) of the sample or in terms of the protein or hemoglobin content.

Step 4: A theoretical unique factor is determined to convert change in absorbance per minute (ΔA/min) to the corresponding units of enzyme activity. This factor is calculated using the following equation:

U/L = ΔA/minute X F; where F= factor

$F = (TV/SV) \times 10^3 / 6.22$ where

TV = Total Volume in mL
SV = Sample Volume in mL
10^3 = converts mL to L
6.22 = millimolar absorbance coefficient

This factor can be programmed into the spectrophotometer and the machine directly converts the change in absorbance at 340 nm (ΔA/min) to activity in U/L. For this assay, with the proper volumes, the factor calculates to be 5144.7.

EXAMPLE WITH MANUAL CALCULATION

The QC Material was assayed for total glutathione peroxidase activity at 25ºC with a cuvette path length of 1 cm using the assay procedure above. Change in absorbance was recorded every 15 sec. The first 40 seconds were not taken into consideration.

Determination of the Reaction rate:

Time (sec)	Absorbance 340 nm (Sample)	Absorbance 340 nm (Blank)
40	0.808	0.746
55	0.775	0.742
70	0.741	0.738
85	0.707	0.735
100	0.674	0.731

Figure 1 shows the slope (rate) of the linear portion of the curve when the absorbance (A_{340}) values are plotted as a function of time (in seconds) in the sample above.

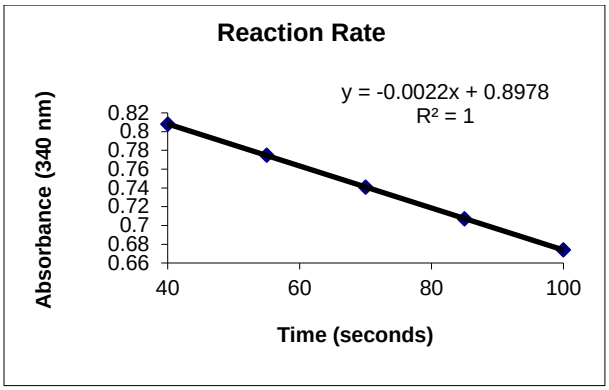

Figure 1

Select highest and lowest points on the linear curve and determine the change in absorbance at 340 nm during the time interval. In this example it is: A_{340} (Time 2)- A_{340} (Time 1)/ T2-T1

0.808-0.674/100 sec-40 sec = 0.134/min

Sample Rate, ΔA_{340}/min= 0.134
Blank Rate, ΔA_{340}/min= 0.015
Net Rate ΔA_{340}/min= 0.12

Calculation of activity

GPx Activity U /L = 1 µmol/min/L = (ΔA_{340}/min)/0.00622 X d X (TV/SV in µl)

For a 1 cm cuvette path length (d) = 0.12/0.00622 X 960/30 = 19.29 X 32 = 617.28 U/L
Unit definition: 1 unit of glutathione peroxidase will form 1.0 µmol NADP+ from NADPH per min at pH 8.0 at 25ºC.

RESULTS

Table 1 shows the precision statistics of this assay for one batch of QC Material, in terms of coefficient of variation (%CV) for two lots of kits. The tests were carried out in 5 days of 10 tests each day done in 2 batches. GPx activity is expressed in terms of U/L. The acceptable GPx activity range for the QC Material with this Kit varies between a lower value of 845.50 and an upper value of 971.35 U/L as measured by the Cobas Fara automated system and a lower value of 749.15 and an upper value of 910.31 U/L done manually.

Precision and Reproducibility of Method

	Intraassay; N=10	Interassay; N=20	Between 5 Days; N=50	Lot to Lot variability N=100
Mean GPx Activity (U/L)	**680.67**	**600.06**	**617.23**	**634.98**
+/- SD	**17.16**	**14.03**	**52.43**	**67.56**
%CV	**2.52**	**2.38**	**8.49**	**10.58**

Table 1

Linearity: Shown below is activity in U/L for the QC material spiked with increasing volume of purified bovine erythrocyte Glutathione Peroxidase enzyme (10,000 U/L stock solution; Sigma). The assay is linear within a change in absorbance range of 0.130 to 0.275 A_{340}/minute which for our QC material spiked with bovine erythrocyte GPx, corresponds to 510 U/L to 1324 U/L enzyme activity respectively. Further experiments have established an accurate measurement of change in absorbance to 0.05 A_{340}/minute corresponding to activity value as low as 261 U/L (results not shown). Accordingly, change in absorbance values below or above the lower and upper limits (0.05 and 0.275 A_{340}/min) indicate the need for use of a more concentrated or more diluted sample.

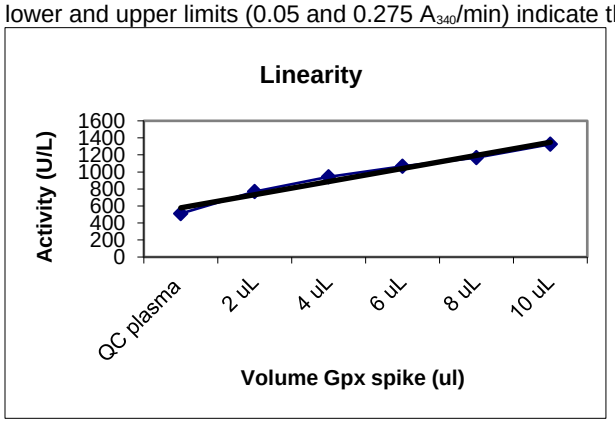

Figure 2

Figure 3 shows the range of dilutions that the Total Glutathione Peroxidase Assay kit can accurately measure GPx activity in human RBC hemolysate. Dilutions of 1:4, 1:8, 1:10,1:20, 1:30, 1:40, 1:50 and 1:100 of a 160 g/L hemoglobin in a RBC hemolysate were tested. Activity is expressed in U/g Hemoglobin. The Kit is effectively accurate over a range of 2.5 fold dilution from 1:20 to 1:50 of the hemolysate that corresponds to 8.05 g/L and 3.22 g/L hemoglobin respectively.

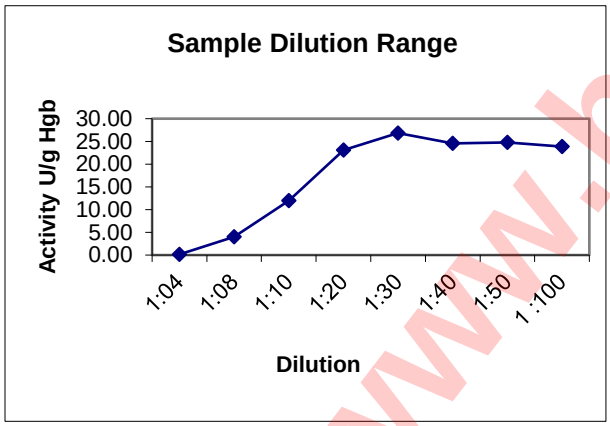

Figure 3

Experiment:

The Total Glutathione Peroxidase Assay Kit was used to calculate total GPx activity from 15 random samples of blood that were obtained commercially. The plasma and red blood cells were separated and appropriate dilutions of the RBC hemolysate were made. The GPx activity for plasma and the RBC hemolysate were calculated both manually and by automation using the Cobas Fara II Chemistry System. * Correlation between the two methods used (manual vs automated) was 0.97 for the plasma and 0.969 for the WRBC.

*** Parameters for the automated system are available upon request.**

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PROCEDURAL FLOW CHART

TURN ON SPECTROPHOTOMETER SET AT 340 nm
↓
SET ASSAY TEMPERATURE AT 25ºC
↓
PREPARE REAGENTS
↓
PREPARE SAMPLES
↓
SET ZERO at 340 nm WITH DI WATER
↓
PIPETTE FOLLOWING REAGENTS INTO A CUVETTE

870 µL REACTION BUFFER
60 µLSTART SOLUTION
30 µL SAMPLE
↓
PIPETTE GENTLY UP AND DOWN
↓
COVER WITH PARAFILM AND TURN GENTLY 2-3 TIMES
↓
PLACE CUVETTE IN CORRECT POSITION IN SPECTROPHOTOMETER
↓
RECORD THE CHANGE IN A_{340} FOR 1 MIN

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