*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® pl•GPx Enzyme Immunoassay™

Assay for Human Plasma Glutathione Peroxidase
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21014

INTRODUCTION

The Analyte

Glutathione peroxidases are selenoenzymes which catalyze the reduction of hydroperoxides (H_2O_2 or ROOH) in the presence of glutathione (GSH). Human blood contains, in addition to Se-GPx in erythrocytes, a plasma-specific glutathione peroxidase (pl•GPx). Glutathione peroxidase is a tetramer of approximately 94-100 kDA,[1-2] with each of the four identical subunits containing an active site with an essential selenocysteine residue. pl•GPx differs from the other glutathione peroxidases by its primary sequence, its glycosylated N-terminal region, and its extra-cellular location. Initially purified from human plasma, pl•GPx has also been found in human milk.[3] It was reported that pl•GPx is mostly synthesized and secreted by renal cells from rat kidney.[4] Another study has shown that a human liver hepatoblastoma cell line (Hep G2) synthesizes and secretes pl•GPx.[5]

Principles of the Procedure

The pl· GPx-EIA™ method is an enzyme-linked immunoassay (ELISA). Samples are incubated in the wells of a sectionable microplate, which have been coated with polyclonal antibodies and are specific for human pl· GPx. These antibodies have been obtained by using a synthetic antigen and have been purified by affinity chromatography.[a]

The presence of pl· GPx is detected by means of a biotinylated-polyclonal antibody to pl•GPx. The final step of the assay is based on the amplification by a biotin-streptavidin coupling in which streptavidin has been covalently linked to alkaline phosphatase. The amount of pl•GPx is quantified by the enzymatic hydrolysis of *p*-nitrophenyl phosphate (pNPP). This requires a microplate reader able to read the absorbance at a wavelength of 405 nm.

REAGENTS

- Sample Diluting Buffer(1) — Tris-HCl buffer, containing bovine casein, NaCl, Tween-20, and sodium azide 0.2%. This solution is used for sample dilution. 3 x 20 mL
- pl•GPx Standard (2) — Approximately 600 ng of purified pl· GPx with bovine serum albumin (BSA) in lyophilized form. 1 vial
- Washing Buffer (3) — Tris-HCl, containing NaCl, Tween-20 and sodium azide 0.1%. 100 mL, 20x concentrated
- Anti-pl•GPx Solution (4) — 75 µL concentrated solution of rabbit polyclonal antibody to pl· GPx in Tris-HCl buffer, containing NaCl, bovine casein, glycerol, and sodium azide 0.1%. 1 microtube
- Streptavidin-PAL (5) — 75 µL concentrated solution of streptavidin-coupled alkaline phosphatase in Tris-HCl, containing NaCl, $MgCl_2$, bovine casein, and sodium azide 0.1%. 1 microtube

[a] US Patent 5861262

- Diluting Buffer (6) — Tris-HCl buffer, containing bovine casein, NaCl, Tween-20, and sodium azide 0.2%. For diluting solutions (4) and (5). 3 x 8 mL
- pNPP Diluting Buffer (7) — Diethanolamine buffer, containing $MgCl_2$ and sodium azide 0.1%. 1 x 20 mL
- Stop Solution (8) — Sodium hydroxide 1 M, containing EDTA. 1 x 20 mL
- pNPP Substrate (9) — Four tablets.
- Microplate — One resealable packet containing 6 x 16 well strips plus frame.
- Plate Sealers — 4 self-adhesive sheets

Materials Required But Not Provided

- Deionized water
- Glass test tubes and beakers
- Adjustable pipettes (100-1000 µL)
- Incubator 37 ± 1 ^{o}C
- Microplate reader capable of absorbance measurements at 405 nm.

Warnings and Precautions

- For *in vitro* use only.
- In case of accidental exposure of skin, eyes, or mucous membranes to Stop Solution (8), wash the exposed area thoroughly with water for 15 minutes.
- The final concentration of sodium azide is less than 0.2%. Before disposal, waste solutions should be diluted in excess of water.
- Human pl· GPx was purified from human plasma and should be handled with standard universal precautions**.** Standard (2) solution should be manipulated with the same precautions as that usually required for other blood products of human origin.

PROCEDURE

Reagent Preparation

Solutions (1), (3), (6), (7), and (8) should be placed at room temperature for 30 minutes before use. Buffers (1) and (6) should be used within 5 days after opening the bottles.

pl•GPx Standard (2)

Prepare a solution of pl•GPx standard (300 ng/mL) by adding 2 mL of Sample Diluting Buffer (1) to the lyophilized protein. This solution may be stored between 2 and 8°C for 24 hours. For longer storage, prepare aliquots immediately and store at –20°C. The pl· GPx standard is stored under vacuum; the bottle should be opened carefully.

Washing Buffer 20x (3)

This solution should be diluted 20 times with water prior to use. Table 1 gives examples of dilutions based on the number of strips used. The diluted Wash Buffer is stable for 5 days at room temperature.

Table 1: Dilution Volumes For Wash Buffer

Number of 16-Well-Strips Used	Required Volume of Wash Buffer	Volume of Concentrated Wash Buffer 20X (3)	Volume of Deionized Water
2	250 mL	12.5 mL	237.5 mL
4	500 mL	25 mL	475 mL
6	1000 mL	50 mL	950 mL

Anti-pl•GPx Solution (4) and Streptavidin-PAL (5)

These solutions should be diluted 250 times with Diluting Buffer (6) prior to use. Examples of some dilutions are described in table 2.

Table 2: Dilution Volumes For Solution (4) And (5)

Number of 16-Well Strips Used	Volume of Diluting Buffer(6)	Volume of Anti-pl· GPx Solution (4) or (5)
2	4 mL	16 µL
4	8 mL	32 µL
6	12 mL	48 µL

pNPP Solution (9)

Fifteen minutes prior to use, dissolve the required number of pNPP tablets (9) in pNPP Diluting Buffer (7). Examples are given in table 3.

Table 3: Dilution Volumes For Pnpp Tablets And Buffer

Number of 16-Well-Strips Used	Volume of pNPP Diluting Buffer (7)	Number of pNPP Tablets (9)
2	5 mL	1
4	10 mL	2
6	15 mL	3

Assay

Microtiter Plate Preparation

- Determine the number of wells required from the number of samples to assay plus the eight levels of standard times the number of replicates you intend to run.
- Use only the number of 16 well strips required. Remove unneeded strips from the frame and place in the resealable foil packet. Store at 2-8°C for future use.
- Pipette 100 µL of sample or standard into each well.
- After assay, keep the frame for future use.

Standard Preparation

- Perform a serial dilution of the 300 ng/ml Standard to the following concentrations: 150, 75, 37.5, 18.75, 9.4, 4.7 ng/ml.
- Add 100µL Standard or sample to the appropriate well.
- Cover the wells with a Plate Sealer and incubate at room temperature for two hours. (± 5 minutes).

Anti-pl· GPx Incubation

- Prepare the required volume of diluted Anti-pl•GPx solution (4) (table 2).
- Empty the microplate.
- Wash wells 5 times with diluted Wash Buffer (3).
- Blot residual liquid on absorbent paper.
- Pipette 100 µL of anti-pl•GPx diluted solution into each well.
- Cover the wells with a Plate Sealer and incubate at room temperature for 1 h. (± 5 min.).

Streptavidin-PAL Incubation

- Prepare the required volume of Streptavidin-PAL solution (5) (table 2).
- Empty the microplate.
- Wash wells 5 times with diluted Wash Buffer (3).
- Blot residual liquid on absorbent paper.
- Pipette 100 µL of Streptavidin-PAL diluted solution into each well.
- Cover the wells with Plate Sealer and incubate at room temperature for 1 h. (± 5 min).

Colorimetric Measurement

- Prepare the required volume of pNPP solution (table 3).
- Stir for 10 minutes.
- Empty the microplate.
- Wash wells 5 times with diluted Wash Buffer (3).
- Blot residual liquid on absorbent paper.
- Pipette 100 µL of prepared pNPP solution in each well.
- Cover the wells with a Plate Sealer and incubate at 37°C until the absorbance of the 300 ng/mL standard reaches 1.5 - 2.0 units (approximately 20 minutes).
- Add 50 µL of Stop Solution (8) to each well.
- Mix and measure the absorbance at 405 nm.

Results and Calculations

The Standard curve is obtained by fitting the standard absorbance at 405 nm to the concentration of pl•GPx by 4-parameter logistic curve fit.

Figure 1: Example of 4-parameter logistic curve fit:

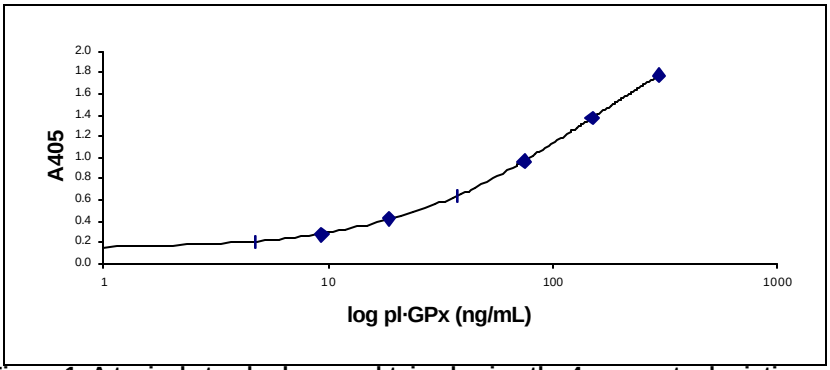

Figure 1: A typical standard curve obtained using the 4-parameter logistic curve fit.

NOTES

Examples of Sample Preparation

- Samples should be diluted, if necessary, using Sample Diluting Buffer (1).
- Dilute human plasma or serum samples approximately 200-300 times with Sample Diluting Buffer (1).
- Samples are stable for 24 hours at 4°C; samples should be stored at -20°C. Avoid repeated freezing/thawing.
- EDTA as an anticoagulant may interfere with pl•GPx EIA™ Assay measurements.

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