Technical Bulletin

Glutathione Peroxidase Cellular Activity Assay Kit

Catalog Number CGP1

Product Description

Glutathione peroxidase (GPx, EC 1.11.1.9) provides a mechanism for detoxification of peroxides in living cells.[1,2] This reaction plays a crucial role in protecting cells from damage by free radicals, which are formed by peroxide decomposition. Lipid components of the cell are especially susceptible to reactions with free radicals, resulting in lipid peroxidation. GPx enzymes reduce peroxides to alcohols using glutathione, thus preventing the formation of free radicals.

GPx enzymes catalyze the reduction of hydrogen peroxide (H_2O_2) and a wide variety of organic peroxides (R-OOH) to the corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent.

Most cellular glutathione peroxidases are tetrameric enzymes consisting of four 22 kDa monomers, each of which contains a selenocysteine moiety in the active site.[2] The selenocysteine participates directly in electron donation to the peroxide substrate and becomes oxidized in the process. The enzyme then uses reduced glutathione as a hydrogen donor to regenerate the selenocysteine. GPx enzymes also exist as non-selenium (non-Se) containing enzymes.[3]

Cellular GPx is present in all tissues; however, various diseases may influence its level. An increase in the level of glutathione peroxidase has been observed in reticulocytes of diabetic rats. The level returned to normal after administration of insulin.[6] A decrease in the level of the enzyme has been observed in patients suffering from diseases such as Favism[7] (a disease associated with extreme hemolytic crisis) or hairy cell leukemia.[8]

This kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH

(β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced).

The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions.[1,6]

$$\text{R-OOH} + 2\ \text{GSH} \xrightarrow{\text{GPx}} \text{R-OH} + \text{GSSG} + H_2O$$

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

GPx is glutathione peroxidase, GR is glutathione reductase, and R-OOH is an organic peroxide.

The reaction is performed at 25 °C and pH 8.0,[10] and is started by adding an organic peroxide, *tert*-butyl hydroperoxide (*t*-Bu-OOH).[11] This substrate is suitable for the assay since its spontaneous reaction with GSH is low and it is not metabolized by catalase. The reaction with *tert*-butyl hydroperoxide measures the amount of selenium-containing glutathione peroxidase activity present.

If the presence of non-Se enzymes is suspected, cumene hydroperoxide can be used as the substrate at a concentration of 0.25–1.0 mM.[4,5] This will measure the total GPx (Se and non-Se enzymes) activity. The difference between the activity observed with cumene hydroperoxide and the *tert*-butyl hydroperoxide activity is the non-Se glutathione peroxidase activity.

Components

Sufficient for 100 tests

- Glutathione Peroxidase Assay Buffer, Catalog Number G8664 — 120 mL
  50 mM Tris HCl, pH 8.0, containing 0.5 mM EDTA
- NADPH Assay Reagent, Catalog Number N5283 — 5 vials
  When reconstituted with 1.25 mL of water, each vial will prepare a solution containing 5 mM NADPH, 42 mM reduced glutathione, and 10 units/mL of glutathione reductase
- Oxidizing Reagent Catalog Number CGP1A — 1 bottle
  70% aqueous solution of *tert*-Butyl Hydroperoxide

Reagents and Equipment Required but Not Provided

- UV/Vis spectrophotometer with thermostated cuvette holder and a kinetic program
- Quartz cuvette
- Glutathione peroxidase, Catalog Number G6137, for use as control enzyme
- Human immunoglobulin G, Catalog Number I4506, and dithiothreitol, Catalog Number D0632, for use with the control enzyme glutathione peroxidase

Precautions and Disclaimer

For Research use only. Not for use in Diagnostic Procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

Store the kit at –20 °C. If daily use of the kit is needed, the assay buffer and *tert*-butyl hydroperoxide may be stored for convenience at 2–8 °C. The components in this kit are stable for 24 months when unopened.

Preparation Instructions

Glutathione Peroxidase Assay Buffer – Bring a suitable aliquot to room temperature. For long term stability of the solution after opening, handle the solution in an aseptic manner.

NADPH Assay Reagent – Reconstitute 1 vial in 1.25 mL of water. Store the solution at 2-8 °C. This reconstituted solution should be used within 3 hours. Each vial is sufficient for at least 20 tests. Do not freeze the solution for reuse.

Oxidizing Reagent Working solution (30 mM *tert*-Butyl Hydroperoxide Solution) – Dilute 21.5 µL of Oxidizing Reagent, Catalog Number CGP1A, to a total volume of 5 ml with water to prepare a 30 mM *tert*-Butyl Hydroperoxide solution.

Glutathione Peroxidase Positive Control (**not supplied**)

The Glutathione Peroxidase Positive Control is used qualitatively to assure the reaction is working. The Glutathione Peroxidase Assay Kit can determine enzyme activity from different biological sources.

Positive control using Catalog Number G6137:

- Dissolve a 100 unit vial of glutathione peroxidase, Catalog Number G6137, in 1 mL of Glutathione Peroxidase Assay Buffer.
- The 100 units/mL solution is diluted 400-fold to a Working Stock Solution of 0.25 units/mL. The dilution to 0.25 unit/mL is made with Glutathione Peroxidase Assay Buffer supplemented with 1 mg/mL IgG, Catalog Number I4506 or equivalent, and 1 mM DTT, Catalog Number D0632 or equivalent.
- Use 20–50 mL of the Working Stock Solution in the reaction to obtain values within the linear range of 0.005-0.02 units per reaction volume of 1 mL. This is equivalent to an absorbance decrease of 0.032-0.13 per minute.
  Note: The IgG is required for stabilization of the enzyme. It is only required in cases of very pure and dilute enzyme solutions.

Positive control using red blood cells:

- Obtain 10 mL of red blood cells, wash twice with 10 mL PBS, centrifuge 3,000 × *g* for 5 minutes at 4 °C. Discard supernatant.
- Resuspend the pellet with 15 mL of a solution containing 1 mM DTT and 5 mM EDTA in ultrapure water.
- Mix gently for 20 minutes at 4 °C.
- Ultracentrifuge the suspended blood at 35,000 rpm for 80 minutes at 4 °C.

As described in the "Sample preparation" section, perform several dilutions of the supernatant for use in the reaction in order to obtain results within the linear range where the absorbance decreases between 0.032 and 0.13 OD per minute

Sample preparation – Dilute crude biological samples in Glutathione Peroxidase Assay Buffer.

Notes:
- Perform several dilutions of the sample for use in the reaction in order to obtain results within the linear range.
- The final concentration of the reagents in the assay mixture is 0.25 mM NADPH, 2.1 mM reduced glutathione, 0.5 unit/mL glutathione reductase, and 300 µM *t*-Bu-OOH.

Procedure

Tissue extracts may contain enzymes that utilize NADPH and skew the results. A blank without Oxidizing Reagent (*t*-Bu-OOH) can be used as a control for these endogenous activities. When liver samples are analyzed, H_2O_2 should be used to start the reaction (0.15–0.30 mM solution) since *t*-Bu-OOH is also a substrate for glutathione S-transferase. In this case, catalase must be blocked by addition of 1 mM NaN_3 in the reaction mixture.[12] In addition, when using H_2O_2 instead of *t*-Bu-OOH, the pH of the Glutathione Peroxidase Assay Buffer should be adjusted to pH 7.0 with HCl. At a pH greater than 7.0 there will be a spontaneous

reaction of hydrogen peroxide with reduced glutathione.

High concentrations of reducing agents such as DTT or 2-mercaptoethanol (>0.1 mM final concentration in the assay system) will depress the measured activity by ~40% at 0.15 mM and up to 70% at 1 mM concentration in the assay. EDTA at 5 mM in the assay will depress the activity by ~50%.

Nonionic detergents such as TWEEN® 20 and Triton™ X-100 that contain high levels of endogenous peroxides will raise the apparent activity. If these detergents are vital to the extraction of the proteins of interest, a low peroxide detergent should be used, such as Catalog Numbers X100PC (Triton X-100), P6585 (TWEEN 20), or P8192 (TWEEN 80).

1. Pipette the volume of Glutathione Peroxidase Assay Buffer indicated in Table 1 into a 1 mL quartz cuvette. Keep the temperature of the assay buffer in the spectrophotometer at 25 °C by using a thermostated cell holder.

Table 1.

Glutathione Peroxidase Reaction Scheme

	GPx Assay Buffer (µL)	NADPH Assay Reagent (µL)	Enzyme (0.25 units/mL) (µL)	Sample (µL)	Oxidizing Reagent Working solution (µL)
Blank	940	50	–	–	10
Positive control	890–920	50	20–50	–	10
Sample	890–930	50	–	10–50	10

2. Add 50 µL of the NADPH Assay Reagent and 10–50 µL of sample or 20–50 µL of enzyme to the cuvette and mix by inversion. The total volume in the cuvette should be 1.00 mL.

3. Start the reaction by addition of 10 µL of the Oxidizing Reagent Working solution. Mix by inversion.

4. Follow the decrease in absorbance at 340 nm using a kinetic program. The following program is recommended:

   Wavelength: 340 nm
   Initial delay: 15 seconds
   Interval: 10 seconds
   Number of readings: 6

5. Calculate the amount of enzyme in the sample.

Calculation

The spectrophotometer should give the A_{340}/min from the reaction automatically. If the absorbance is measured manually, calculate this value for the blank, positive controls, and all samples.

The activity of Glutathione Peroxidase in the sample can be calculated using the formula:

Activity per extract (mmol/min/mL = Units/mL)

$$\frac{\Delta A_{340} \times DF}{6.22 \times V}$$

Where:

ΔA_{340} = $A_{340}/min_{(blank)} - A_{340}/min_{(sample)}$
6.22 = ε^{mM} for NADPH
DF = dilution factor of sample before adding to reaction
V = sample volume in mL

Unit definition: 1 unit of glutathione peroxidase will cause the formation of 1.0 μmol of $NADP^+$ from NADPH per minute at pH 8.0 at 25 °C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and Oxidizing Reagent (*tert*-butyl hydroperoxide).

References

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