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UC Davis - Glutathione Peroxidase

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We use this protocol and it's working

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Abstract

Summary:

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxides, 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. Each subunit contains a selenocysteine in the active site which participates directly in the two-electron reduction of the peroxide substrate. The enzyme uses glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine. The Cayman Chemical Glutathione Peroxidase Assay Kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GPx activity in the sample. 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.

Materials

MATERIALS

Assay Kit **Cayman Chemical Company** **Catalog #**703102

Buffer

Standard

Co-Substrate Mix

Cumene Hydroperoxide

Note:

Cayman Chemical **RRID:SCR_008945**

1 **Background or Non-enzymatic Wells** - add 120 µl of Assay Buffer and 50 µl of co-substrate mixture to three wells.

2 **Positive Control Wells (bovine erythrocyte GPx) -** add 100 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 20 µl of diluted GPx (control) to three wells.

3 **Sample Wells** - add 100 µl of Assay Buffer, 50 µl of Co-Substrate Mixture, and 20 µl of sample to three wells. To obtain reproducible results, the amount of GPx added to the well should cause an absorbance decrease between 0.02 and 0.135/min. When necessary, samples should be diluted with Sample Buffer or concentrated with and 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****.(Below)(page 14)*

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.

4 Initiate the reactions by adding 20 µl of 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.*

7 **Caculation**

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

a. Plotting 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 13 using bovine erythrocyte GP_x) -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 or non-enzymatic wells and subtract this rate from that of the sample wells.

3. Use the following formula to calculate the GP_X activity. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373μM^{-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 ºC.

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

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