Glutathione Peroxidase Activity Colorimetric Assay Kit

(Catalog # BN00975; 100 reactions; Store kit at –20° C)

I. Introduction:

Glutathione Peroxidase (GPx, EC 1.11.1.9) family of enzymes play important roles in the protection of organisms from oxidative damage. GPx converts reduced glutathione (GSH) to oxidized glutathione (GSSG) while reducing lipid hydroperoxides to their corresponding alcohols or free hydrogen peroxide to water. Several isozymes have been found in different cellular locations and with different substrate specificity. Low levels of GPx have been correlated with free radical related disorders. In Assay Genie's Glutathione Peroxidase Activity Assay, GPx reduces Cumene Hydroperoxide while oxidizing GSH to GSSG. The generated GSSG is reduced to GSH with consumption of NADPH by GR. The decrease of NADPH (easily measured at 340 nm) is proportional to GPx activity. The assay can be used to measure all of the glutathione dependent peroxidases in plasma, erythrocyte lysates, tissue homogenates, and cell lysates with a detection sensitivity of ~ 0.5 mU/ml of GPx in samples.

II. Kit Contents:

Components	BN00975	Cap Code	Part Number
GPx Assay Buffer	50 ml	NM	BN00975-1
NADPH (lyophilized)	1 vial	Blue	BN00975-2
Glutathione Reductase	1 vial	Green	BN00975-3
Glutathione (GSH; lyophilized)	1 vial	Brown	BN00975-4
Cumene Hydroperoxide	1 vial	Yellow	BN00975-5
GPx Positive Control (lyophilized)	1 vial	Red	BN00975-6

III. Storage and Handling:

Store the kit at -20°C, protect from light. Warm the assay buffer to room temperature before use. Briefly centrifuge vials before opening. Read the entire protocol before performing the assay.

IV. Reagent Reconstitution and General Consideration:

NADPH: Reconstitute with 0.5 ml dH_2O to get a 40 mM NADPH solution.
GR: Dilute with 0.22 ml Assay Buffer.
GSH: Reconstitute with 0.22 ml Assay Buffer.
Cumene Hydroperoxide: Dilute with 1.25 ml Assay Buffer. Mix well
GPx Positive Control: Reconstitute with 100 µl Assay Buffer.

All the solutions are stable for at least 1 week at 4 °C and 1 month at -20 °C.
Ensure that the assay buffer is at room temperature before use. Keep samples, GR mix solution and GPx Positive Control on ice during the assay.

V. Glutathione Reductase Activity Assay:

1. Sample Preparations:

Homogenize 0.1 g tissues, 10^6 cells, or 0.2 ml erythrocytes on ice in 0.2 ml cold assay buffer; Centrifuge at 10,000 x g for 15 min at 4 °C; Collect the supernatant for assay and store on ice. Serum can be tested directly. Keep samples at -80 °C for storage. Add 2 - 50 µl of the samples into a 96-well plate; bring the volume to 50 µl with Assay Buffer. We suggest testing several doses of your sample to make sure the readings are within the standard curve range.

2. NADPH Standard Curve:

Dilute 25 µl of the 40 mM NADPH solution into 975 µl dH_2O to generate 1 mM NADPH standard. Add 0, 20, 40, 60, 80, 100 µl of the 1 mM NADPH Standard into 96-well plate in duplicate to generate 0, 20, 40, 60, 80,100 nmol/well standard. Bring the final volume to 100 µlwith Assay Buffer. Measure O.D. 340 nm to plot the NADPH Standard Curve.

3. Positive Control (optional) and Reagent Blank:

For Positive Control use 5 - 10 µl of the GPx Positive Control into the desired well(s) and adjust to 50 µl with Assay Buffer. Add 50 µl of Assay Buffer into a well (s) as a Reagent Control (RC).

4. Reaction Mix:

For each well, prepare 40 µl Reaction Mix:

33 µl Assay Buffer
3 µl 40 mM NADPH solution
2 µl GR solution
2 µl GSH solution

Add 40 µl of the Reaction Mix to each test samples, Positive Control (s) and RC(s) mix well, and incubate for 15 minutes to deplete all GSSG in your sample**.** Add 10 µl Cumene Hydroperoxide Solution to start GPx reaction. Mix well. Measure OD 340 nm at T1 to read A1, measure OD 340 nm again at T2 after incubating the reaction at 25 °C for 5 min (or longer if the GPx activity is low) to read A2, protect from light. $\Delta A_{340\ nm}$ = [(Sample_A1-Sample_A2) – (RC_A1 – RC_A2)]

Notes:

A. Measure the OD 340 nm before adding Cumene Hydroperoxide. Add more NADPH if the Sample OD at 340 nm is lower than 1.0 to ensure there is enough NADPH in the reaction system. 1 µl of 40 mM NADPH will give ~ 0.5 OD at 340 nm.
B. If A1 reading is too low (< 0.7), it means either too much GPx or too much GSSG presence in the sample. You may need to dilute the samples, or remove GSSG from your sample using methods, such as dialyzing the sample or using spin filters to remove GSSG.
C. It is essential to read A1 and A2 in the reaction linear range. It will be more accurate if you read the reaction kinetics. Then choose A1, A2, in the reaction linear range.

5. Calculation:

Plot NADPH standard Curve. Apply the ΔA_{340nm} to the NADPH standard curve to get NADPH amount B.

$$\text{GPx Activity} = \frac{B}{(T2 - T1) \times V} \quad \text{X Sample dilution = nmol/min/ml = mU/mL}$$

Where: **B** is the NADPH amount that was decreased between T1 and T2 (in nmol).
T1 is the time of first reading (A1) (in min).
T2 is the time of second reading (A2) (in min).
V is the pretreated sample volume added into the reaction well (in ml).

Unit Definition: One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25°C.

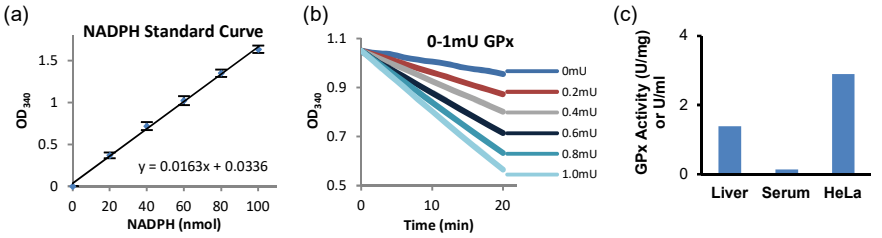

Figure: (a) NADPH Standard Curve. (b) Measurement of GPx activity using purified enzyme. (c) GPx Activity was measured using rat liver lysate (23 µg), human serum (1 µl) and HeLa cell lysate (16 µg). Assays were performed following the kit protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		