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NWLSS™ Glutathione Peroxidase Assay

Product NWK-GPX01
For Research Use Only



Simple assay kit for quantitative measurement of glutathione peroxidase in biological samples such as whole blood, tissue homogenates and cell lysates.



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Table of Contents**End-User Notes:**

Section	Page
Introduction	3
Intended Use	3
Test Principle	3
General Specifications	4
Kit Contents	4
Required Materials Not Provided	4
Required Instrumentation	4
Warnings, Limitations, Precautions	5
Storage Instructions	5
Assay Preparation	5
Reagent Preparation	6
Sample Handling/Preparation	6
Assay Protocol	8
Data Analysis	8
Performance Details	10
Things To Note	11
References	12
Procedure Checklist	12
Statement of Limited Warranty	13
End-User Notes	13

End-User Notes:**Introduction:**

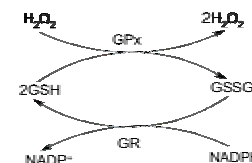
Glutathione Peroxidase (GPx) (EC 1.11.1.9) enzymes belong to a family of selenoproteins whose function is to catalyze the reduction of various peroxides. Four types of GPx (GPx-1 to 4) have been identified in mammalian species. GPx-1 is the classic intracellular form while GPx-2, GPx-3 and GPx-4 are gastrointestinal, plasma and phospholipid hydroperoxidase forms; respectively. Three of the forms are tetramers composed of 4 identical 22 kDa monomers while GPx-4 is monomeric. The monomeric subunits of GPx-1 to 3 each have the amino acid selenocysteine at the active site of the enzyme. The best characterized and most heavily researched form is GPx-1, the intracellular form, sometimes also called cellular GPx (cGPx).

Intended Use:

The NWLSS™ Glutathione Peroxidase assay has been designed to quantify the enzyme activity of glutathione peroxidase. The assay is expected to be most useful for measurement of cellular GPx (GPx-1).

Test Principle:

The NWLSS™ Glutathione Peroxidase Assay is an adaptation of the method of Paglia and Valentine¹. Glutathione Peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β -nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPx is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPx concentration



GPx activity is reported as units based on the definition:

1 unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of 1.0 μ mole GSH to GSSG, per minute at 25 °C, pH 7.0.

The assay has been designed for use in 96 well microplate format although it can also be performed using disposable cuvettes on a spectrophotometer in laboratories not equipped with a microplate reader. The method has been validated on whole blood, red blood cell lysates and tissue homogenates. Plasma glutathione peroxidase will also react in the assay. However, researchers are cautioned that due to the large amount of GPx-1 contained in erythrocytes, even a small amount of hemolysis will result in an overestimation of plasma GPx-3 activity.

General Specifications:

Format:: 2 X 96 wells

Number of tests: Triplicate = 62
Duplicate = 94

Specificity: Glutathione Peroxidase

Sensitivity: LLD = 5.0 mU/mL by plate reader
LLD = 1.0 mU/mL by spectrophotometer

Kit Contents:

NADPH Reagent β -Nicotinamide adenine dinucleotide phosphate and
GSH, reduced; 2 bottles powder.

NADPH Diluent: Glutathione Reductase in buffer with stabilizer;
2 x 6 mL.

Assay Buffer Phosphate buffer with EDTA, pH 7.0; 1 x 125 mL

H₂O₂ Reagent 3% Hydrogen Peroxide

Microplates: 2 Each

Required Materials Not Provided:

Adjustable pipettes capable of transferring 20 μ L to 1.0 mL volumes.
A multi-channel or repeater pipette (recommended)

Optional Materials:

Glutathione peroxidase (Sigma catalog # G 6137) for control purposes.

Required Instrumentation:

Microplate reader with kinetics capability at 340 nm.
25 °C temperature control is recommended to achieve consistency with
GPx unit activity definition but is not required.

Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

End-User Notes:

References:

1. D.E. Paglia, and W.N. Valentine (1967) *J. Lab. Clin. Med.* 70, 158-169; Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase.
2. Sigma product # G 6137 publication.

Procedure Checklist for Plate Reader:

- Process the sample and place on ice
- Bring GPx reagents to ambient temperature
- Setup microplate reader
 - Wavelength = 340 nm
 - Mode = Kinetic
 - Reaction Time = 5 Minutes
 - Read Interval = 30 seconds
 - Temperature = 25 °C (optional)
- Dilute samples as appropriate with Assay Buffer and place on ice.
- Reconstitute NADPH Reagent with NADPH Diluent as specified.
- Add 50 µL diluted samples to appropriate wells of microplate
- Add 50 µL Working NADPH to each well
- Add 50 µL Working H₂O₂ to each well
- Equilibrate 1 minute
- Place microplate in plate reader and begin 340 nm measurements.
- Calculate results
- Return reagents to 2-8 °C.

Warnings, Limitations, Precautions:**NADPH**

β-Nicotinamide adenine dinucleotide phosphate, reduced form (CAS 2646-71-1) is irritating to the eyes, respiratory system and skin. Target organs: nerves, liver. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing.

Interference

Reducing agents, such as cysteine, β-mercaptoethanol or dithiothreitol may interfere at concentrations greater than 0.1 mM.

Intrinsic absorbance at 340 nm (e.g. hemoglobin) must be <1.2

Excess GSSG may result in overestimation of GPx. A high initial rate followed by stabilization is evidence of this. GSSG may be removed by dialysis, desalting or by delaying recording or by simply using the linear portion of the curve obtained. Note that extremely high levels of GSSG would exhaust the NADPH in the reaction.

NADPH consuming enzymes may cause overestimation of GPx activity. If a concern, then employ a sample blank.

Polyethoxynonyl detergents (Tween-20, Triton X-100) may contain peroxides.

Storage Instructions:

Upon receipt, store the reagents at 2-8°C. Do not use components beyond the expiration date printed on the label.

All reagents should be brought to room temperature (18-25°C) prior to use.

Assay Preparation**Plate Reader Setup Recommendations**

Wavelength: 340 nm

Duration: 5 minutes

Interval: 30 seconds

Data Reduction: Linear regression

Temperature: 25 °C (Optional)

Reagent Preparation:*NADPH Diluent*

This reagent is supplied ready to use.

NADPH Reagent

1. Add the entire contents of one NADPH Diluent bottle to one NADPH bottle.
2. Secure cap on vial containing the reconstituted NADPH and mix briefly by inverting the bottle.

Note: During the assay, *Working NADPH* should be kept on ice to prolong the useful life of the reagent. NADPH cocktail solution is usable for up to 3 days (72 hours) if stored at 2-8°C in the original container.

H₂O₂ Reagent

1. Dilute 3% hydrogen peroxide as supplied 1:100 (e.g. .02 mL 3% H₂O₂ to 2 mL *Assay Buffer* and mark as *Dilution 1*).
2. Further dilute *Dilution 1* 3:100 (e.g. 0.3 mL *Dilution 1* to 10 mL *Assay Buffer* and mark as *Working H₂O₂*

Sample Handling/Preparation:

The multi-disciplinary interest in measuring GPx has resulted in a myriad of sample types and experimental conditions and is beyond the scope of this product insert to describe sample processing in detail for each case. However, general guidelines are provided below for representative sample types. If additional information is required, please contact NWLSS to discuss the particular sample under investigation.

Whole Blood

Collect samples using EDTA, heparin, citrate or ACD anticoagulant. Store at 2-8°C. DO NOT FREEZE. Whole blood must be diluted in water to lyse the cells then additionally diluted in Assay Buffer to achieve the appropriate pH.

1. Dilute whole blood 1:9 in deionized H₂O.
2. Dilute the whole blood lysate 1:9 in Assay buffer.

Whole blood may be assayed directly after dilution in Assay Buffer and is essentially a measure of GPx-1 (cGPx). Since plasma GPx (GPx-3) is typically present in much lower concentrations than cGPx, it does not contribute significantly to the measured activity.

Plasma

Plasma GPx (GPx-3) will react in the assay however users are cautioned that a very small amount of hemolysis during plasma isolation will result in a very large presence of cellular GPx (GPx-1) in the sample causing over-estimation of plasma GPx activity.

Performance Details (continued):*Dynamic Range:*

As can be seen on the plot of ΔA_{340} vs time (page 8), very high GPx concentrations can result in an underestimated rate from the linear regression over the 5 minute interval. Samples with greater than 65 mU/mL should be additionally diluted with assay buffer and retested. Based on the departure from linearity at higher concentrations, the dynamic range of the assay is estimated as:

1 mU/mL - 65 mU/mL by spectrophotometer
5 mU/mL - 65 mU/mL by plate reader

Accuracy

GPx enzyme was added to an erythrocyte lysate. Average recovery upon dilution was 92% of expected value.

Stability

All unopened reagents are stable until the expiration date stated on the package label when stored at 2-8°C.

Reconstituted NADPH Reagent is usable for up to 3 days (72 hours) if stored at 2-8°C if stored sealed and uncontaminated in the original bottle.

Things to Note:*Unit Definition:*

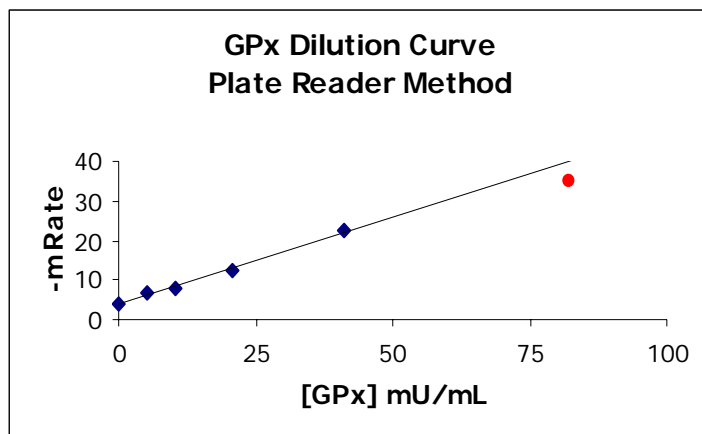
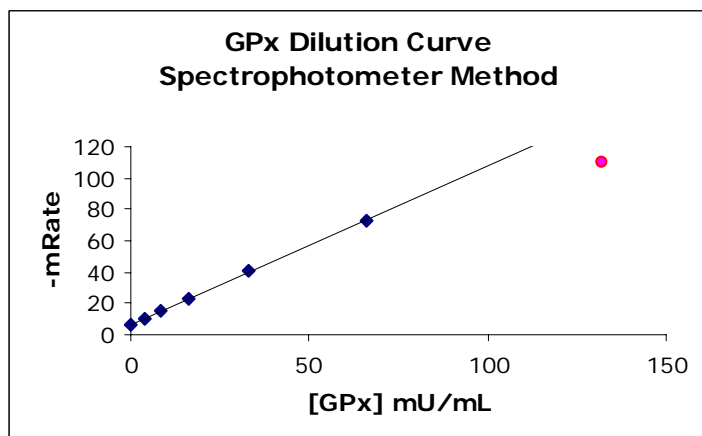
Note that the NWLSS™ unit definition for GPx activity is consistent with Sigma Chemical's definition and method of testing for their commercially available glutathione peroxidase (product # G 6137). Therefore, the Sigma product used as a positive control with the NWLSS™ NWK-GPX01 assay should return results consistent with the values indicated on the product label.

Reaction Stoichiometry and Comparison to Published Data:

The reaction stoichiometry of GSH to GSSG requires 2 moles GSH for every GSSG and thus every mole NADPH oxidized as well. Since GPx quantification is based on the NADPH extinction coefficient, users will notice the delta rate must be multiplied by 2 to account for the GSH to GSSG reaction stoichiometry. It may appear that a simple division by 2 will generate data comparable to published data of the NADPH to NADP+ definition. Researchers are cautioned however that GPx activity varies with temperature (approximately 5% percent activity change may occur per degree temperature variance) and pH (activity at pH 8.8 is around 10X that of pH 7.0 activity). For these reasons inter-study data can be compared for qualitative similarities among cohorts but comparison of actual numerical data is not recommended.

Performance Details:*Linearity on Dilution*

GPx recovery is linear upon dilution as shown in the following figures for the spectrophotometer method and plate reader method.

*Sensitivity*

Defined as 3.2 standard deviations from zero

Sensitivity is estimated to be:

1 mU/mL by spectrophotometer

5 mU/mL by plate reader.

Tissue

Contaminating blood contains significant concentrations of erythrocytes and thus cGPx. If possible, blood should be removed by perfusion with an appropriate buffer, such as phosphate buffered saline containing heparin prior to freezing or homogenization. It is preferable to harvest fresh tissues, homogenize and assay same day. However, if this is not possible, it is recommended that samples be harvested, sectioned as appropriate and flash frozen for later homogenization and assay. Clarify homogenates by centrifugation and store the supernatant on ice. Tissue homogenates may be frozen -70°C and assayed at a later date if necessary. The GPx concentration can be normalized to the wet weight of the tissue sample or to the protein concentration of the homogenate. As a general guideline NWLSS recommends a 20-40% w/v homogenization be performed in Tris Buffer containing 5 mM EDTA, 1 mM DTT or 2ME, pH 7.5 to help guard against oxidation of GPx. Users should target 0.1-1.0 mg/mL protein concentration in the reaction. A typical homogenate might have a protein concentration of around 20-40 mg/mL requiring an additional 1/10 dilution in Assay Buffer prior to testing. This additional dilution is necessary to reduce the concentration of DTT or 2ME in the final assay reaction.

Cultured Cells

Prepare a cell suspension of approximately 10^6 cells per mL in an appropriate buffer and homogenize or disrupt the cells by sonication. Clarify the homogenate by centrifugation and store the supernatant on ice. The GPx concentration can be normalized to the cell number or to the protein concentration of the homogenate. If the mass of the cell pellet/sample is known, samples may be prepared according to instructions given for tissues. It is recommended that a trial assay with a representative sample be tested to determine if the samples are within the dynamic range of the assay. If the test sample is below the sensitivity of the standard procedure, please contact NWLSS for assistance.

Erythrocytes

Collect blood with EDTA, Citrate, ACD, or heparin anticoagulant and isolate erythrocytes by centrifugation. Wash the cells 2-3 times in ice cold saline (0.15M NaCl). Lyse the cells by suspending in 4X packed cell volumes of cold, deionized water. Lysates should be placed on ice until assayed. If samples cannot be assayed within 2-3 hours they can be stored at -70°C until date of assay.

It is recommended that the sample be diluted to achieve a 0.2-0.5 mg/mL hemoglobin concentration. In males at 4,500 ft, average hemoglobin = 16.4 g/dL; therefore the lysate will be ~ 65 mg/mL. In this case, the lysate should be diluted 30-40x in Assay buffer and 50 μL of the diluted sample added to the well for testing.

Assay Protocol:*Standard Procedure for Microplate Assay*

1. Bring all reagents to room temperature.
2. Remove microplate from plastic bag.
3. Add 50 μ L of diluted sample (or controls if present) to wells.
4. Add 50 μ L of *Working NADPH* to each well.
5. Add 50 μ L of *Working H₂O₂* to each well.
6. Wait 1 minute, monitor A₃₄₀ for 5 minutes with a recording interval of every 30 seconds.
7. Calculate GPx activity from the net rate.

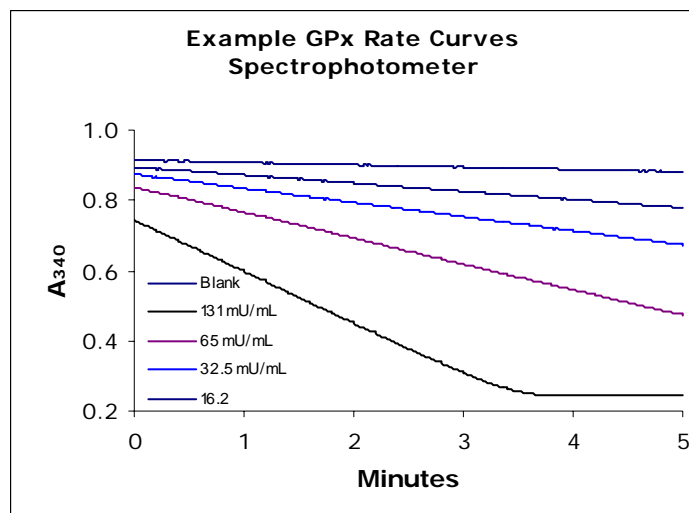
Note: Reagent additions for spectrophotometer method are dependant on cuvette minimum working volume specifications however 200 μ L diluted sample and reagent additions are recommended.

Data Analysis:

The following example shows the expected behavior and results of GPx determination using the standard method with the NWLSS™ Glutathione Peroxidase assay.

Reaction Rate:

The decrease in 340 nm absorption is a linear function of time. As reactants become exhausted, the rate begins to decrease. In the figure below, the 131mU/mL sample becomes non-linear at 3.5 minutes resulting in under-estimation of GPx activity (see GPx Dilution curves later in this publication).

**Data Analysis:**

Note: When determining rates on a plate reader recording should be started after 1 minute delay to allow for proper equilibration of reagents to occur.

Calculate GPx Concentration Using the NADPH Absorption Coefficient:

The GPx concentration, expressed as mU/mL, is calculated using the GPx activity definition. For the spectrophotometer the equation is...

$$[GPx] = \frac{2(mRate_s - mRate_b) \cdot V_{R_{xm}} \cdot df}{6.22 \cdot V_s}$$

...where

$mRate_s = -1000 \times \Delta A_{340} / \text{min of sample}$

$mRate_b = -1000 \times \Delta A_{340} / \text{min of blank}$

6.22 = NADPH 340 nm millimolar absorption coefficient at 1 cm pathlength.

$V_{R_{xm}}$ = Volume of Reaction Mixture

V_s = Volume of Sample

2 = Correction for 2 moles GSH oxidized to 1 mole GSSG per mole NADPH oxidized.

df = Sample dilution factor

For the microplate application, the pathlength is proportional to the volume of the reaction mixture. The pathlength is 0.44 cm and the effective millimolar absorption coefficient is 2.74 for the microplates supplied in the kit. For the microplate application, the equation is...

$$[GPx] = \frac{2(mRate_s - mRate_b) \cdot V_{R_{xm}} \cdot df}{2.74 \cdot V_s}$$