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ab219926 Glutathione Peroxidase Activity Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of Glutathione Peroxidase activity in biological samples.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Glutathione Peroxidase Activity Assay Kit (Fluorometric) (ab219926) provides a simple method to measure Glutathione Peroxidase activity in cell lysates.

This assay is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by Glutathione Peroxidase (GPx). The generated GSSG is recycled to its reduced state GSH by glutathione reductase (GR) and NADPH:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPX}} \text{R-O-H} + \text{GSSG} + H_2O$$

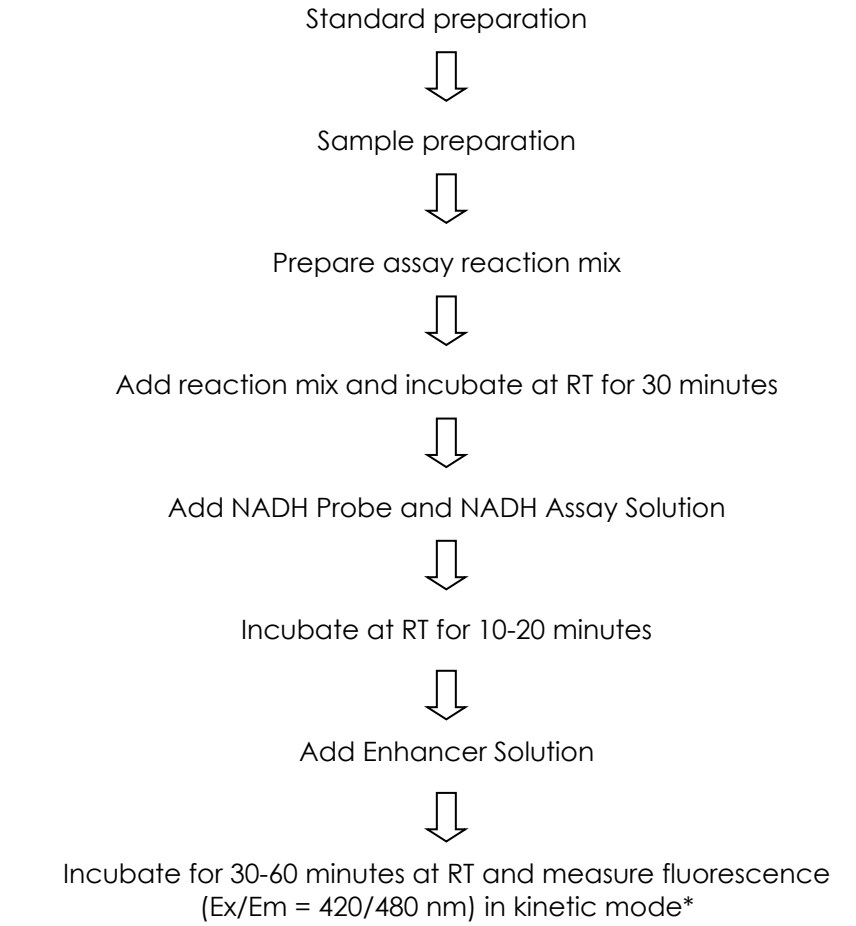
$$\text{GSSG} + \text{NAPDH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The reaction product, $NADP^+$, can be specifically monitored using our newly developed proprietary NADP sensor, which reacts with NADP only to generate a fluorescent product. The signal, which is proportional to the GPx activity present in the sample, can be measured with a fluorescence microplate reader at Ex/Em = 420/480 nm.

This assay can detect activity from as low as 1.25 mU/mL Glutathione Peroxidase in solution. The assay can be performed in a convenient 96-well or 384-well plate format and is easily adapted to automation without a separation or wash step.

Glutathione peroxidase (GPx) is an enzyme family with peroxidase activity to protect the organism from oxidative damage. GPx plays an important role in reducing organic hydroperoxides such as lipid hydroperoxides to their corresponding alcohols, or reducing free hydrogen peroxide to water. It therefore guards against oxidative damage to the cell membranes and other oxidant-sensitive sites in the cell. It has been noticed that altered GPx levels correlate with lesions caused by many comment and complex diseases. GPx level is measured in biological samples as a potential indicator for the potential treatment of cancer, diabetes, neurodegenerative and cardiovascular diseases.

2. Protocol Summary



**For kinetic mode detection, incubation time given in this summary is for guidance only*

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C desiccated in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Assay Buffer	10 mL	-20°C	-20°C/ 4°C
Enhancer Solution	3.5 mL	-20°C	-20°C
Enzyme Mix	2 vials	-20°C	-20°C
Glutathione Peroxidase Standard (0.5 U)	1 vial	-20°C	-20°C
GSH (3 mg)	1 vial	-20°C	-20°C
NADP Assay Solution	5 mL	-20°C	-20°C
NADP Probe	5mL	-20°C	-20°C
Substrate	11 µL	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 420/480 nm
- PBS
- Double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96-well solid black plate
- Cell scraper (for adherent cells)
- (Optional) Mammalian Cell Lysis Buffer 5X (ab179835)
- BSA

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer (10 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C or 4°C.

9.2 Enhancer Solution (3.5 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze-thaw cycles. Use within two months.

9.3 Enzyme Mix (lyophilized):

Dissolve the content of one vial in 5 mL of Assay Buffer and mix thoroughly by pipetting up and down.

Δ Note: this amount is enough for 1 x 96 well plate. If you are not going to run a full plate, aliquot mix and store at - 20°C. Be aware however that sensitivity might decrease. Avoid repeated freeze-thaw cycles. Use within two months.

9.4 Glutathione Peroxidase Standard (0.5 U):

Dissolve GPx Standard in 50 µL of ddH_2O or 1X PBS buffer and mix thoroughly by pipetting up and down to make a 10 U/mL GPx standard stock solution.

Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze-thaw cycles. Use within two months.

9.5 GSH (3 mg):

Dissolve GSH in 100 µL of ddH_2O and mix thoroughly by pipetting up and down. Label this component **100X GSH stock solution**. Aliquot so that you have enough volume to perform the desired number of assays. Store at - 20°C. Use within two months.

9.6 NADP Assay Solution (5 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at - 20°C. Use within two months.

9.7 NADP Probe (5 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot so that you have enough volume to perform the desired number of assay. Store at - 20°C. Use within two months.

9.8 Substrate (11 µL):

Dissolve Substrate in 100 µL of ddH_2O and mix thoroughly by pippeting up and down. Label this component **100X substrate stock solution**. Aliquot so that you have enough volume to perform the desired number of assays. Store at - 20°C. Use within two months.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 0.1 U/mL (100 mU/ml) GPx standard by diluting 10 µL of the 10 U/mL GPx standard (Step 9.4) with 990 µL of 1XPBS + 0.1% BSA. Mix well by pipetting up and down.

10.2 Using the 0.1 U/mL GPx standard, perform 1:1.5 serial dilutions in PBS + 0.1% BSA as described in the table in a microplate or microcentrifuge tubes:

Standard #	Sample to dilute	Volume standard in well (µL)	PBS +0.1% BSA Buffer (µL)	End activity GPx in well (mU/mL)
1	Step 10.1			100
2	Std #1	200	100	66.7
3	Std #2	200	100	44.4
4	Std #3	200	100	29.6
5	Std #4	200	100	19.8
6	Std #5	200	100	13.2
7	Std #6	200	100	8.78
8 (blank)	0	200	100	0

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

Diluted GPx standard solution is unstable, and should be used within 4 hours.

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.
- Samples prepared by other protocols can be used as well for this assay. Do not use RIPA buffer as it will interfere with the assay. If you have your samples ready, please skip this section and proceed to Assay Procedure section.

11.1 Cell lysates:

Δ Note: For ease of use, mammalian adherent or suspension cells lysates can be easily prepared using Mammalian Cell Lysis Buffer 5X (ab179835). Follow product protocol and proceed to Section 12.

11.1.1 Harvest the number of cells necessary for each assay (initial recommendation: 2-5 x 10^5 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend or scrape cells in 100 µL of cold PBS.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Centrifuge 5 minutes at 4°C at 13,000 x*g* in a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Keep on ice.

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- Prepare all reagents and samples as directed in the previous sections.
- The protocol describe in this section is for 1 x 96-well plate. To perform the assay in a 384-wp, scale down volumes by half.

12.1 Set up reaction wells:

- Blank control = 50 µL PBS + 0.1% BSA.
- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with PBS).

12.2 GPx assay reaction:

12.2.1 Prepare GPx assay mixture by adding 50 µL 100X GSH stock solution (Step 9.5) and 50 µL 100X substrate stock solution (Step 9.8) to the Enzyme mix solution (Step 9.3). Mix well.

Δ Note: GPx assay mixture is not stable; please use it promptly. It is not recommend storing unused GPx assay mixture.

12.2.2 Add 50 µL of GPx assay mixture into each well to make the total volume of 100 µL/well.

12.2.3 Incubate the reaction at room temperature for 30 minutes, protected from light.

12.3 NADH assay reaction:

12.3.1 Add 20 µL NADP Probe into each reaction well and mix well by pipetting up and down.

12.3.2 Add 20 µL NADP Assay Solution into each reaction well. Mix well by pipetting up and down.

12.3.3 Incubate the reaction at room temperature for 10-20 minutes, protected from light.

12.3.4 Add 15 µL Enhancer into each well to make the total assay volume of 155 µL/well.

12.4 Measurement:

12.4.1 Monitor fluorescence increase at Ex/Em = 420/480 nm on a microplate reader in kinetic mode, every 2-3 minutes, for at least 30-60 minutes, protected from light.

Δ Note: Incubation time depends on the Glutathione Peroxidase activity in the samples. We recommend measuring fluorescence in a kinetic mode and choosing two time points (T1 and T2) to calculate the GPx activity of the samples.

13. Data Analysis

– Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1 Determination of reaction rate:

13.1.1 Subtract the fluorescence value of the blank (Standard #8) from all standard and sample readings. This is the corrected fluorescence.

13.1.2 Plot the standard readings as function of the GPx activity (mU/mL) and draw the line of the best fit (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Measurement of Glutathione Peroxidase activity in the sample:

13.2.1 Interpolate Glutathione Peroxidase activity (mU/mL) on the test samples from the standard curve data using the trend line equation.

13.2.2 Calculate GPX activity in your sample as follows:

$$GPX\ Activity = B * \left(\frac{Vwell}{Vsample}\right) * D$$

Where:

B = GPX activity in sample well calculated from standard curve.

Vwell = total volume of well after reaction.

Vsample = original sample volume added into the reaction well.

D = sample dilution factor if sample is diluted to fit within the standard curve range.

Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

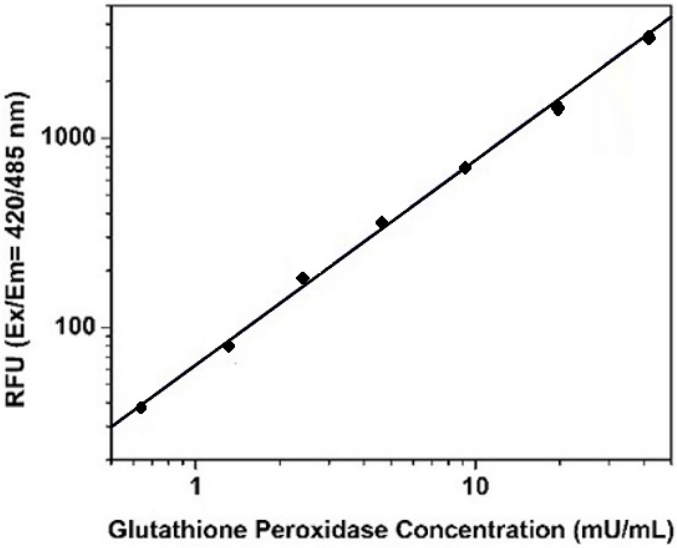


Figure 1. Typical Glutathione Peroxidase dose response curve. Fluorescence was measured on a solid black 96-well plate using a Gemini microplate reader (Molecular Devices). As low as 1.25 mU/mL GPx can be detected with 30-60 minutes incubation.

14. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare reagents and aliquot; get equipment ready.
- Prepare GPx standard dilution [40-0.625 mU/mL]
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL) and samples (50 µL)
- Prepare GPx assay mixture: 50 µL 100X GSH stock solution + 50 µL 100X substrate stock solution + 5 mL Enzyme mix solution
- Add 50 µL) GPx assay mixture to each well
- Incubate at RT for 30 minutes
- Add 20 µL NADH probe and 20 µL NADH solution to each well
- Incubate at RT for 10-20 minutes
- Add 15 µL enhancer solution to each well
- Monitor fluorescence increase at Ex/Em 420/480 nm on a microplate reader in kinetic mode for 30-60 minutes at RT protected from light.

15. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Fluorometric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

16. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)