Peroxidase Activity Colorimetric/Fluorometric Assay Kit

(Catalog #BN00985; 100 reactions; Store kit at –20ºC)

I. Introduction:

Peroxidases (EC number 1.11.1.x) are a large family of enzymes that typically catalyze a reaction of the form: ROOR' + electron donor (2 e^-) + $2H^+$ → ROH + R'OH. For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or alternately redox-active cysteine or selenocysteine residues. Assay Genie's Peroxidase Assay Kit provides a convenient colorimetric and fluorometric means to measure the peroxidase activity in biological samples. In the presence of Peroxidase, the GenieRed Probe reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. The resorufin is quantified by colorimetric (λ_{max} = 570nm) or fluorometric methods (Ex/Em = 535/587 nm). The assay is simple, direct, highly sensitive and high throughput-ready. The detection limit is 0.1 mU per well via colorimetric or 0.01 mU per well via fluorometric method, based on our unit definition.

II. Kit Contents:

Components	BN00985	Cap Code	Part No.
Assay Buffer	25 ml	WM	BN00985-1
GenieRed Probe (in DMSO)	0.2 ml	Red	BN00985-2
H_2O_2 Substrate (0.88 M)	0.1 ml	Yellow	BN00985-3
HRP Positive Control	1 vial	Green	BN00985-4

III. Storage and Handling:

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

IV. Reagent Preparation and Storage Conditions:

- **H_2O_2 Substrate:** Dilute H_2O_2 Substrate to 12.5 mM by adding 5 μl of H_2O_2 substrate (0.88 M) to 347 μl Assay Buffer. The diluted H_2O_2 Substrate is stable for one day at 4°C and one month at -20°C.
- **HRP Positive Control:** Add 1 ml assay buffer into lyophilized HRP to prepare HRP solution. The HRP solution is stable for one day at 4°C and one month at -20°C.
- **GenieRed Probe:** Before use, briefly warm at 37°C for 1-2 min to completely melt DMSO solution, mix well. Store at –20°C.

V. Peroxidase Assay Protocol:

1. **Sample Preparation:** Collect cell culture supernatant, serum, plasma, urine, and other biological fluids. Centrifuge test samples for 15 minutes at 1000 x g within 30 min of collection to remove particulate pellet. Assay immediately or aliquot and store the samples at -80°C. Avoid repeated freeze-thaw cycles. Add 2-50 µl samples into each well and adjust the final volume to 50 µl with Assay Buffer.
2. **Standard Curve Preparations:**

   For Colorimetric Assay: Dilute H_2O_2 substrate solution to 0.1 mM by adding 10 µl of H_2O_2 substrate solution (12.5 mM) to 1240 µl Assay Buffer, mix well. Add 0, 10, 20, 30, 40, 50 µl into a series of wells in duplicate and adjust the final volume to 50 µl with Assay Buffer to generate 0, 1, 2, 3, 4, 5 nmol/well of H_2O_2 standard.

   For Fluorometric Assay: Dilute H_2O_2 substrate solution to 0.01 mM by adding 100 µl of H_2O_2 substrate solution (0.1 mM) to 900 µl Assay Buffer, mix well. Add 0, 10, 20, 30, 40, 50 µl into a series of wells in duplicate and adjust the final volume to 50 µl with Assay Buffer to generate 0, 100, 200, 300, 400, 500 pmol/well of H_2O_2 standard.
3. **Standard Curve Measurement:** Dilute HRP positive control solution 1:199 in Assay Buffer. For each well, prepare a total 50 μl Reaction Mix containing 2 µl GenieRed Probe and 48 µl diluted HRP positive control solution, mix well. Incubate for 5 min and measure the OD at 570 nm or RFU at Ex/Em = 535/587 nm in a micro plate reader.
4. **Positive Control Preparation:** Use 1 µl of the diluted positive control solution into the desired well(s) and adjust the final volume to 50 µl with Assay Buffer**.**
5. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50 μl Reaction Mix:

   46 μl Assay Buffer
   2 μl GenieRed Probe solution
   2 μl H_2O_2 Substrate solution

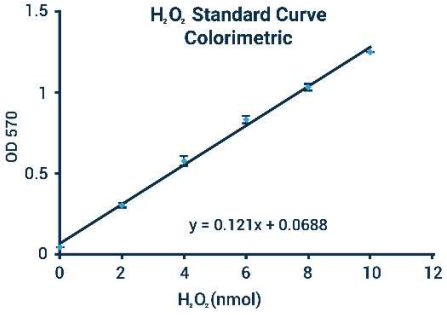
   Add 50 μl of the Reaction Mix to each test samples and HRP positive control. Mix well; incubate the mix for 3 min at 37°C.
6. **Measurement:** Measure OD 570 nm (A_0) for colorimetric assay or Ex/Em = 535/587 nm (R_0) for fluorometric assay. Incubate for another 30 min to 2 hr at 37°C to measure OD at 570 nm (A_1) or fluorescence at Ex/Em = 535/587 nm (R_1) again, incubation times will depend on the peroxidase activity in the samples. We recommend measuring the OD or fluorescence in a kinetic method (preferably every 3 – 5 min) and choose the period of linear range, which falls within H_2O_2 Standard Curve to calculate the peroxidase activity of the samples.
7. **Calculation:** Plot the H_2O_2 Standard Curve. Calculate the Peroxidase activity of the test samples: $\Delta A = A_1 - A_0$ or $\Delta RFU = R_1 - R_0$, apply the ΔA or ΔRFU to the H_2O_2 Standard Curve to get B nmol of H_2O_2 generated by peroxidase in the given time.

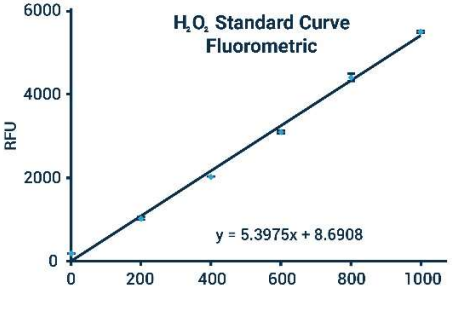
Peroxidase Activity = $\frac{\mathbf{B}}{\mathbf{T} \times \mathbf{V}}$ **× Sample Dilution Factor = nmol/min/ml = mU/ml**

Where: **B** is the H_2O_2 amount from standard Curve (in nmol)
T is the time incubated (in min).
V is the sample volume added into the reaction well (in ml).

Unit Definition: One unit of Peroxidase is the amount of enzyme that will oxidize 1.0 μ mol of H_2O_2 per min at 37°C.

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 or White plates (if background low); Luminescence: White plates ; Absorbance: 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 standard	• Avoid pipetting small volumes
	• 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
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• 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.		