Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit

(Catalog #K265-200; 200 reactions; Store kit at -20°C)

I. Introduction:

Hydrogen Peroxide is a reactive oxygen metabolic byproduct that serves as a key regulator for a number of oxidative stress-related states. Functioning through NF-κB and other factors, hydroperoxide-mediated pathways have been linked to asthma, inflammatory arthritis, atherosclerosis, diabetic vasculopathy, osteoporosis, neuro-degenerative diseases, Down's syndrome and immune system diseases. **BioVision's Hydrogen Peroxide Assay Kit** is a highly sensitive, simple, direct and HTS-ready colorimetric and fluorometric assay for measuring H_2O_2 in biological samples. In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacts with H_2O_2 to produce product with color (λ_{max} = 570 nm) and red-fluorescent (Ex/Em = 535/587 nm). The kit can perform 200 reactions by fluorometric method or 100 reactions by colorimetric method. The detection limit can be as low as 2 pmol/well (or 40 µM) of H_2O_2 in the sensitive fluorometric assay.

II. Kit Contents:

Components	K265-200	Cap Code	Part Number
H_2O_2 Assay Buffer	25 ml	WM	K265-200-1
OxiRed™ Probe (in anhydrous DMSO)	0.2 ml	Red	K265-200-2A
HRP	1 vial	Green	K265-200-4
H_2O_2 Standard (0.88 M)	0.1 ml	Yellow	K265-200-5

III. Storage and Handling:

Warm the assay buffer to room temperature (RT) before use. Briefly centrifuge vials prior to opening. Read the entire protocol before performing the assay.

IV. Reagent Reconstitution and General Consideration:

OxiRed™ Probe: Ready to use as supplied. Briefly warm at 37°C to melt the frozen DMSO. The OxiRed™ Probe solution is stable for 1 week at 4°C and 1 month at -20°C.

HRP: Dissolve in 220 µl H_2O_2 Assay Buffer, pipetting up and down. The HRP solution is stable for 1 week at 4°C and 1 month at -20°C.

V. Hydrogen Peroxide Assay:

1. Sample Preparation:

Collect the cell culture supernatant, serum, plasma, urine and other biological fluids (contains 0.8 - 6 µM H_2O_2). Centrifuge for 15 min at 1000 x g within 30 min of collection. Remove the particulate pellet. Samples, especially those such as culture medium, tissue lysate or plasma should be filtered through 10 kDa spin columns (BioVision, Cat# 1997-25) to remove all the proteins then kept at -80°C for storage. It is recommended to assay the samples(s) immediately or aliquot and store at -80°C. Avoid repeated freeze-thaw cycles. Add 2 - 50 µl samples into each well. Bring the volume to 50 µl with H_2O_2 Assay Buffer.

2. H_2O_2 Standard Curve:

For the Colorimetric Assay: Dilute 10 µl 0.88 M H_2O_2 Standard into 870 µl dH_2O to generate a 10 mM H_2O_2 Standard. Then dilute 10 µl of the 10 mM H_2O_2 Standard into 990 µl dH_2O to generate a 0.1 mM H_2O_2 Standard. Add 0, 10, 20, 30, 40, 50 µl of the 0.1 mM H_2O_2 Standard into 96-well plate in duplicate to generate 0, 1, 2, 3, 4, 5 nmol/well H_2O_2 Standard. Adjust the volume to 50 µl/well using H_2O.

For the Fluorometric Assay: Dilute 100 µl of the 0.1 mM H_2O_2 Standard into 900 µl dH_2O to generate a 10 µM H_2O_2 Standard. Add 0, 10, 20, 30, 40, 50 µl of the 10 µM H_2O_2 Standard into a 96-well plate in duplicate to generate 0, 0.1, 0.2, 0.3, 0.4, 0.5 nmol/well H_2O_2 Standard. Adjust the volume to 50 µl/well using H_2O.

3. Reaction Mix:

Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50 µl Reaction Mix:

Colorimetric Assay	Fluorometric Assay
46 µl Assay Buffer	48 µl Assay Buffer
2 µl OxiRed™ Probe solution	1 µl OxiRed™ Probe solution
2 µl HRP solution	1 µl HRP solution

Add 50 µl of the Reaction Mix to each test samples and H_2O_2 Standard wells. Mix well. Incubate at RT for 10 min.

*For a more sensitive assay, you can dilute the Standard 10 fold further, decrease the OxiRed™ amount to 0.2 µl and HRP amount to 0.4 µl per well,. This will decrease the fluorescence background and detects as low as 2 pmol/well (or 40 µM) H_2O_2.

4. Measure OD (570 nm) or fluorescence (Ex/Em = 535/587 nm) in a micro-plate reader.

5. Calculation:

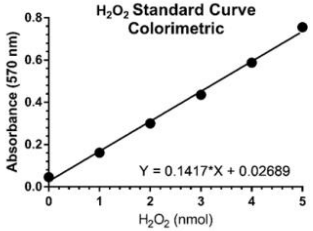
Correct background by subtracting the value derived from the 0 nmol H_2O_2 control from all sample and Standard readings (Note: The background reading can be significant and must be subtracted from sample readings). Plot the H_2O_2 Standard Curve. Apply your sample readings to the Standard Curve. H_2O_2 concentrations of the test samples can then be calculated,

C=Sa/Sv (pmol/µl or µM),

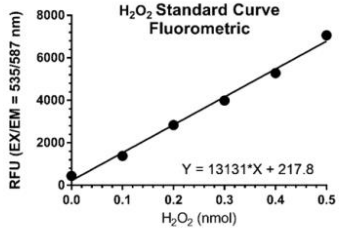
Where: **Sa** is the sample amount from your Standard Curve (in pmol),

Sv is sample volume (µl).

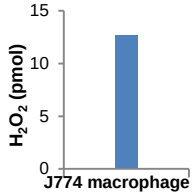
(a)



(b)



(c)



Figures: a) Colorimetric Standard Curve. b) Fluorometric Standard Curve and c) Quantification of H_2O_2 in murine macrophage J774 cells. ~3.8X10^6 cells were lysed in 380 µl of Assay buffer. Cell lysate was centrifuged at 10,000xg for 2 min. and collected supernatant was filtered through a 10 kDa MW spin columns (Cat. # 1997). 10 µl was used to measure H_2O_2. Assay was performed following the kit protocol.

VI. RELATED PRODUCTS:

Glutathione Reductase Assay Kit	Glutathione Peroxidase Assay Kit
Colorimetric Glutathione Detection Kit (GSH, GSSG and Total)	ApoGSH Glutathione Detection Kit Glutathione Kit
GST Colorimetric Assay Kit	GST Fluorometric Assay Kit
Acid Phosphatase Assay Kit	Triglyceride Assay Kit
Phosphate Fluorescence Assay Kit	ADP/ATP Ratio Assay Kit
NAD/NADH Quantification Kit	Phosphate Colorimetric Assay Kit
	NADP/NADPH Quantitation Kit

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 ; 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
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• 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, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till 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.		