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Northwest
Life Science Specialties, LLC

Premier Products for Superior Life Science Research

NWLSS™ Hydrogen Peroxide Assay

Product NWK-HYP01
For Research Use Only

Simple assay kit for quantitative measurement of hydrogen peroxide in biological samples such as urine, cell culture supernatants or other fluids where hydrogen peroxide may be present.

nwkhyp01rev052305

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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.

Notes:

Procedure Checklist

__ Process the sample and place on ice

__ Bring all reagents to room temperature.

__ Setup microplate reader or spectrophotometer
Wavelength = 595 nm or 560 respectively
Mode = Endpoint

__ Dilute samples as appropriate and place on ice

__ Remove microplate from plastic bag.

__ Prepare *Working XO:Fe Reagent*

__ Prepare Calibrator dilutions

__ Add 5 uL catalase to Sample *blank* wells.

__ Add 5 uL deionized H_2O to Sample *test* wells and calibrator wells.

__ Add 20 µL of diluted sample or calibrators to wells.

__ Agitate the plate and incubate for 5 minutes at room temperature.

__ Add 200 µL of *XO:Fe Reagent* to each well.

__ Incubate 45 minutes at room temperature.

__ Read plate at 560-595 nM.

__ Plot standard curve using linear regression analysis.

__ Calculate sample H_2O_2 concentrations by comparing sample absorbance to standard curve obtained.

__ Return reagents to 2-8°C.

Introduction:

Hydrogen Peroxide (H_2O_2) is a highly reactive species normally produced in cells by both enzymatic and non-enzymatic pathways. The reaction of superoxide dismutase (SOD) with superoxide radicals within cells and in surrounding areas results in the uncontrolled formation of H_2O_2. Granulocytes also produce H_2O_2 via metabolic processes as an immunological response to bacteria and other foreign invaders. While the metabolic production of H_2O_2 by granulocytes is controlled at the immunologic level, the production of H_2O_2 as a byproduct of oxidative stress is not well regulated and thus cells must rely on the enzymes catalase and glutathione peroxidase to convert H_2O_2 into harmless water and oxygen. As a byproduct of the SOD oxidative stress response and as an integral part of normal inflammatory response to infection, understanding the impact of H_2O_2 on various model systems has become an important facet of understanding many pathophysiological processes.

Intended Use:

The NWLSS™ NWK-HYP01 Assay is intended for the quantitative determination of hydrogen peroxide specifically when used with the supplied catalase enzyme as directed.

Test Principle:

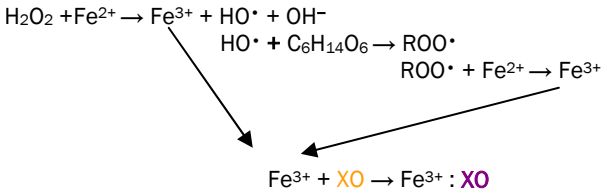
The NWLSS™ NWK-HYP01 assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by peroxides (ROOH where R=H in the case of hydrogen peroxide).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-}$$

The Fe^{3+} then forms a complex with an indicator dye xylenol orange (3,3'-bis[N,N-di(Carboxymethyl)-aminomethyl]-o-cresolsulfone-phthalein, disodium salt, XO) causing an increase in absorbance at 560-590 nm measurable as a purple colored complex.

$$Fe^{3+} + XO \rightarrow Fe^{3+}:XO$$

Sorbitol acts to amplify the color intensity as a function of the original peroxide content of the sample by reacting with hydroxyl radicals to create additional Fe^{3+} for the color producing reaction with Xylenol Orange.



General Specifications:

Format: 2 X 96 wells

Number of tests: 86 duplicate sample tests without catalase blank
43 duplicate sample tests with catalase blank

Specificity: Hydrogen Peroxide

Sensitivity: LLD = Approximately 1.5 μM using either microplate or spectrophotometer method.

Kit Contents:

Xylenol Orange: Xylenol Orange with Sorbitol in water; 90 mL bottle

Fe Reagent: Fe^{2+} in 2.5 M H_2SO_4; 2 mL vial

Catalase Reagent 1 Vial

H_2O_2 Reagent 520 mM hydrogen peroxide with preservative

Microplates: 4 Each

Required Materials Not Provided:

Pipettes capable of transferring 5, 25, 50, 100 and 500 µL volumes.
A multi-channel or repeater pipette (recommended).

Required Instrumentation:

Microplate reader with endpoint capability at 560-595 nm.

Warnings, Limitations, Precautions:

H_2O_2 standards are light sensitive and should be protected from direct exposure to light sources.

EDTA or other iron chelators will interfere in the assay and should be strictly avoided when considering blood collection and/or sample processing buffers.

High levels of antioxidants may interfere with sorbitol signal amplification thus causing underestimation of sample H_2O_2 content.

Concentrations > 100 mM of some sugars (e.g. glucose, sucrose, fructose and mannitol) may cause increased color development resulting in overestimation of sample H_2O_2 content.

Performance Details:

Stability

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

Sensitivity: 1.4 μM when performed in cuvettes and absorbance at 560 nm measured using a spectrophotometer.

1.8 μM when performed in a 96 well plate and absorbance at 595 measured using a plate reader.

Dynamic Range: Approximately 1.5-100 μM

Things to Note:

For spectrophotometer measurement adhere to 10 parts XO:Fe working reagent to 1 part sample. Reagent additions will be dependent on spectrophotometer/cuvette minimum volume requirements.

Measurement of hydrogen peroxide without the catalase sample blank will yield more tests per kit however researchers are cautioned that any substance capable of oxidizing Fe^{2+} or the presence of endogenous Fe^{3+} in samples will result in overestimation of hydrogen peroxide content.

References:

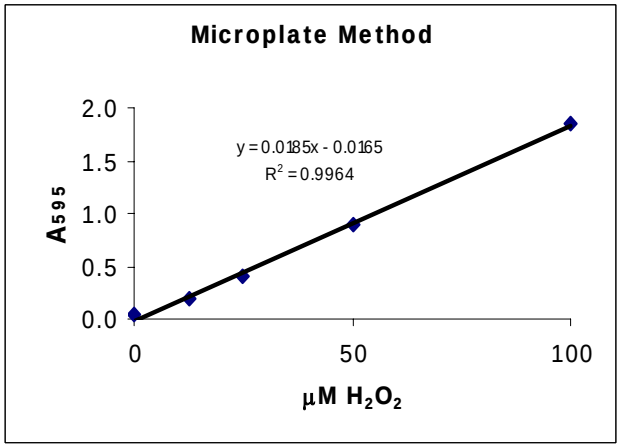
1. Jiang, Z.Y.; Woollard, A.S.; Wolff, S.P. Hydrogen peroxide production during experimental protein glycation. *FEBS* **268**(1):69-71; 1990

2. Wolff, S. P. *Methods Enzymol.* **233**, 182-189; 1994

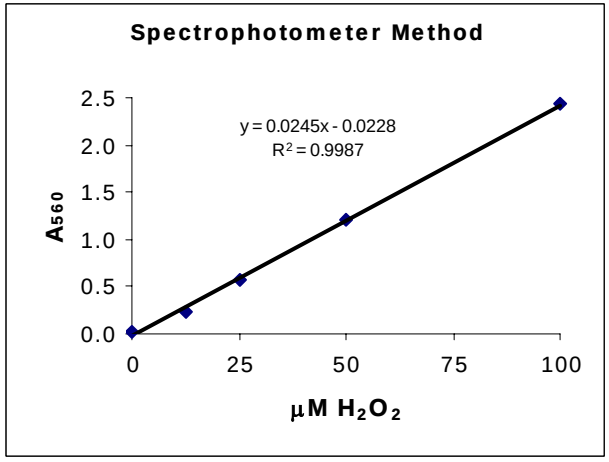
3. Halliwell, B.; Gutteridge, J.M.C. *Free Radicals in Biology and Medicine*. Oxford University Press, New York; 1999.

4. Deiana, L; Carru, C; Pes, G; Tadolini, B; Spectrophotometric measurement of hydroperoxides at increased sensitivity by oxidation of Fe2+ in the presence of xylenol orange. *Free Radic Res.* Sep; **31**(3):237-44; 1999.

Data Analysis:
The following examples show the expected behavior and results of H_2O_2 determination using the standard method with the NWLSS™ Hydrogen Peroxide assay using either a spectrophotometer or plate reader.



For this example: $$[H_2O_2] = \frac{ABS_{net} + 0.0165}{0.0185}$$



For this example: $$[H_2O_2] = \frac{ABS_{net} + 0.0228}{0.0245}$$

Storage Instructions:
Upon receipt, store the reagents at 2-8ºC. Do not use components beyond the expiration date printed on the label.

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

Assay Preparation
Spectrophotometer Setup Recommendations:
Endpoint Wavelength: 560 nm

Data Reduction: Linear regression

Plate Reader Setup Recommendations
Endpoint Wavelength: 560-595 nm

Data Reduction: Linear regression

Reagent Preparation:
Working Xylenol Orange/Fe (XO:Fe) Reagent
1. Dilute 1 part *Fe Reagent* with 100 parts *Xylenol Orange.* Label as **Working XO:Fe.**

Note: Working XO:Iron reagent can be used for up to 5 days if stored at 4 ºC. A new standard curve must be run on each day of assay as increased color development will be evident based on some oxidation of Fe^{2+} to Fe^{3+} during storage of Working XO:Iron solution.

H_2O_2 Calibrator
1. Dilute hydrogen peroxide standard as supplied as follows:
   20uL of 520 mM standard to 1 mL dH_20 to yield 10.2 mM, then…
   20uL of 10.2 mM standard to 1 mL dH_20 to yield 200 uM then…
   Serially dilute 200 uM Standard in tubes containing dH_20 to yield 100, 50, 25 and 12.5 uM assay calibrators. Aliquot 1 mL dH_20 to an additional tube to create the zero calibrator.

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Sample Handling/Preparation:

The multi-disciplinary interest in measuring H_2O_2 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.

Cell Culture Supernatant

Harvest media to a clean tube and centrifuge to clarify. Clarified supernatant should be assayed immediately else frozen at ≤ 70 ºC for later assay.

Urine

Harvest urine to a clean tube and centrifuge to clarify. Clarified urine samples should be assayed immediately else frozen at ≤ 70 ºC for later assay.

Tissue

Quantification of H_2O_2 in experimental tissue or cell homogenates may be possible however this application has not yet been validated. Users are cautioned that "normal" tissues contain the enzymes catalase and/or glutathione peroxidase at concentrations that normally act to reduce H_2O_2 to very low levels.

Assay Protocol:

Standard Microplate Procedure

1. Bring all reagents to room temperature.
2. Remove microplate from plastic bag.
3. Prepare *Working XO:Fe Reagent*
4. Prepare Calibrator dilutions
5. Add 5 uL catalase to Sample *blank* wells.
6. Add 5 uL deionized water to Sample *test* wells and calibrator wells.
7. Add 20 µL of diluted sample or calibrators to wells as appropriate.
8. Agitate the plate and incubate for 5 minutes at room temperature.
9. Add 200 µL of *XO:Fe Reagent* to each well.
9. Incubate 45 minutes at room temperature.
10. Read plate at 560-595 nM.
11. Plot standard curve using linear regression analysis.
12. Calculate sample H_2O_2 concentrations by comparing sample absorbance to standard curve obtained.

For specific H_2O_2 measurements:

$ABS_{net} = ABS_{560\text{-}595}$ {Test Sample} - $ABS_{560\text{-}595}$ {Sample Blank}

Suggested Cuvette Method for Spectrophotometer:

Catalase addition to sample *blank* wells and deionized water addition to sample *test* and calibrator wells is the same as the microplate method (5 µL). Sample and *Working XO:Fe Reagent* requirements for the cuvette method will be dependent on spectrophotometer/cuvette minimum volume requirements. In any case adhere to 10 parts XO:Fe working reagent to 1 part sample. For example:

1. Bring all reagents to room temperature.
2. Set up necessary cuvettes or reaction tubes.
3. Prepare *Working XO:Fe Reagent.*
4. Prepare Calibrator dilutions.
5. Add 5 µL catalase to Sample *blank* wells.
6. Add 5 µL deionized water to Sample *test* wells and calibrator wells.
7. Add 50 µL of diluted sample or calibrators to wells as appropriate.
8. Agitate the plate and incubate for 5 minutes at room temperature.
9. Add 500 µL of *XO:Fe Reagent* to each well.
10. Incubate 45 minutes at room temperature.
11. Measure 560 nM absorbance.
12. Plot standard curve using linear regression analysis.
13. Calculate sample H_2O_2 concentrations by comparing sample absorbance to standard curve obtained.

For specific H_2O_2 measurements:

$ABS_{net} = ABS_{560}$ {Test Sample} - ABS_{560} {Sample Blank}