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NWLSS™ Lipid Hydroperoxide Assay

Product NWK-LHP01
For Research Use Only

Simple assay kit for measurement of lipid hydroperoxides (LOOH); an early measure of lipid peroxidation in plasma, tissue homogenates and cell lysates.

Note: This kit is not suitable for use with metal chelators such that EDTA treated plasma is not measurable using this method.

Table of Contents

Introduction:

Lipids are a varied group of water insoluble compounds which function as energy storage molecules, structural components of biological membranes, enzyme cofactors, intracellular messengers, and other critical biological functions. All lipids are derivatives of fatty acids.[1]

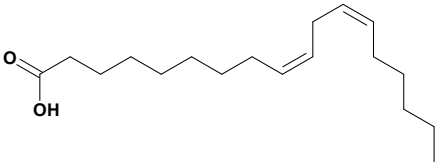


Figure 1. Linoleic Acid (cis,cis-9,12-octadecadienoic acid)

Fatty acids are carboxylic acids containing aliphatic chains of 4-36 carbons. Saturated fatty acids contain no carbon-carbon double bonds, monounsaturated fatty acids have one -C=C- bond while polyunsaturated fatty acids (PUFA) contain 2 or more -C=C- bonds.[1]

Lipid hydroperoxides (LOOH) are the result of oxidation of fatty acids by any species sufficiently reactive to abstract hydrogen from a methylene group, forming a carbon-centered radical that reacts with molecular oxygen to form the lipid peroxide shown in Figure 2.

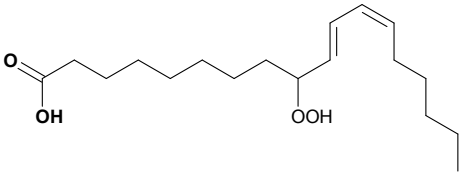


Figure 2. Oxidized linoleic acid or 9(S)-hydroperoxyoctadeca-10E,12Z-dienoic acid.

Lipid peroxidation occurs in two distinct steps; initiation and propagation.

Introduction (continued):

Initiation:

Allylic hydrogens possess a weakened H-C bond and as such are especially prone to abstraction, forming a carbon centered radical.

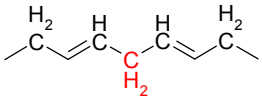


Figure 3: An allylic hydrogen (red) is defined as a methylene hydrogen with an adjacent carbon-carbon double bond. When the hydrogen is between two carbon-carbon double bonds it is a bis-allylic hydrogen.

The carbon-centered radical can then react with molecular oxygen to produce the lipid peroxyl radical. This radical in-turn abstracts hydrogen to form a lipid hydroperoxide (**Figure 4**).

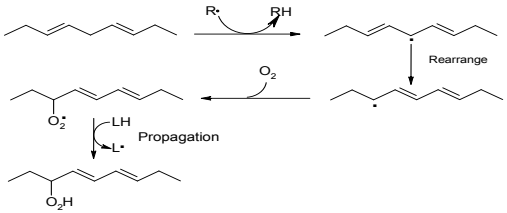


Figure 4. Oxidation of polyunsaturated fatty acids.

Propagation:

As shown in Figure 4, the lipid peroxyl radical can then abstract hydrogen from another lipid molecule. The resulting radical (LO• or L•) can then continue a free radical chain reaction.[2] Iron chelates (DNA-Fe, ATP-Fe, etc.), heme iron (hemoglobin, myoglobin, cytochrome c, etc.) and oxidized and reduced copper all react with LOOH to form LOO•, facilitating propagation of lipid peroxidation.

Intended Use:

The NWLSS™ NWK-LHP Assay is intended for the quantification of Lipid Hydroperoxides; an early measure of lipid peroxidation in plasma, tissue homogenates and cell lysates.

Test Principle:

The NWLSS™ Lipid Hydroperoxide Assay is based on the reaction of LOOH with ferrous iron to form ferric iron and the subsequent reaction of ferric iron with 3,3′-Bis[N,N-bis (carboxymethyl)aminomethyl]-o-cresolsulfonephthalein (Xylenol Orange) to form a chromagen with measurable absorbance at 560 nm.

The basic reactions are as follows:
In acidic medium, LOOH and other oxidants (e.g., hydrogen peroxide, *t*-butyl hydroperoxide and cumene hydroperoxide) will oxidize ferrous iron (Fe(II), Fe^{2+}) to ferric iron (Fe(III), Fe^{3+}).

$$LOOH + Fe^{2+} \longrightarrow LO\bullet + OH^- + Fe^{3+}$$

Figure 5: Oxidation of ferrous iron by lipid hydroperoxide.

While still under acidic conditions, ferric iron, produced by the oxidation of ferrous iron by LOOH and its derivative radical species, forms a complex (XOF) with Xylenol Orange that absorbs between 500-600 nm.

$$Fe^{3+} + \text{Xylenol Orange} \longrightarrow XOF \quad ABS_{560} \uparrow$$

Figure 6: Oxidation of ferrous iron by lipid hydroperoxide.

The slope of the regression of A_{560} vs concentration of 9(S)-HPODE (hydroperoxyoctadecadienoic acid) is equal to the molar absorption coefficient ($E_{560} = 33{,}700\ M^{-1} \bullet cm^{-1}$) of the XOF complex.

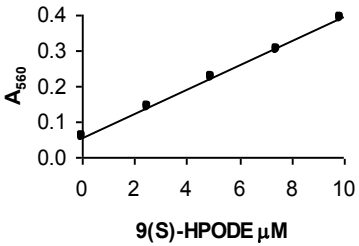


Figure 7: Increasing concentrations of authentic 9(S)-HPODE added to Xylenol Orange:Fe reagent forms the XOF complex and a linear curve when measured at 560 nm and plotted as [LOOH] against A_{560}.

Test Principle (continued):

The calculation of LOOH concentration is based on the equation:

$$[\text{LOOH}] = \frac{A_{560}}{\varepsilon_{560}}$$

with various blanks and calibrator factors added in as shown under "Data Analysis", page 11.

Correcting for sample iron content:

Endogenous iron content in many biological samples will also react with XOF and can result in the overestimation of LOOH concentration. This is corrected by creating a "reduced sample blank" using a suitable reducing reagent. The reagent Tris(2-carboxyethyl)phosphine (TCEP) is supplied for this purpose. A "Sample Blank (SB)" must be determined in addition to the untreated sample in each case.

Correcting for possible H_2O_2 in the sample:

The XOF iron complex[3] is an indirect measurement of all hydroperoxides (including both LOOH and H_2O_2) in the sample. For best possible LOOH specificity, it is necessary to treat samples that may contain H_2O_2 with catalase (provided).

Correcting for variable recovery:

NWLSS provides a calibrator for use when assaying complex biological samples. The calibrator is useful in performing one or more of the following experiments to improve the estimate of LOOH in a given sample.

Internal calibration to measure sample LOOH recovery (recommended).

Verify that the reagent set is performing by recovery of an LOOH control.

Generate a calibration curve relative to 9(S)-HPODE (optional).

General Specifications:

Format:	Spectrophotometric Cuvette
Number of tests:	100
Specificity:	Lipid Hydroperoxides
Sensitivity:	LLD = 0.3μM in reaction mix LLD = 1.9μM in original sample

Kit Contents:

Xylenol Orange:	1 X 8 mL
Iron Reagent:	1 X 8 mL
Catalase Reagent: (ready to use)	1 X 1 mL
TCEP Reagent: (ready to use)	1 X 1 mL
BHT Reagent:	1 Vial
BHT Solvent:	1 X 2.5 mL
Calibrator (9(S)-HPODE): (ready to use)	1 X 1 mL

Required Materials Not Provided:

- Methanol, recently opened bottle recommended
- Pipettes: (5 µL, 10 µL, 50 µL, 100 µL and 500 µL volumes)
- Repeater pipettes – highly recommended
- Containers (10 mL – 300 mL with closure)
- Semi-microcuvette (working volume at least 500 µL)
- Vials, polypropylene, 1-2 mL with closure (o-ring) or equivalent

Required Instrumentation:

Spectrophotometer with 560 nm capability

Warnings, Limitations, Precautions:

Known Interferents:

Metal Chelators: The XOF reaction will be quenched in the presence of iron chelators such as ethylenediaminetetraactic acid (EDTA). Therefore, they cannot be present in the sample.

Possible Interferents:

Endogenous Fe^{3+}: Endogenous Fe^{3+} conjugates to XO; therefore, a sample blank in which the LOOH in the sample is reduced is required for samples suspected of having iron present. This can be accomplished by treating the sample blank with a reducing agent to reduce LOOH[4]

Hemoglobin: The NWLSS™ Lipid Hydroperoxide method uses an MeOH deproteination step which results in the removal of most proteins, including hemoglobin. Therefore, hemoglobin is not considered an interferent.

Warnings, Limitations, Precautions (continued):

Possible Interferents (continued):

Ascorbic Acid: Higher concentrations (300 µM range) may cause overestimation of sample LOOH and is not correctable using calibration.

Noted interference is less at lower concentrations (70 µM range) and is correctable using calibration.

High GSH Concentrations in the 500 µM range were found to cause overestimation of sample LOOH and is not correctable using calibration.

Uric Acid: Concentrations in the range of 250 µM were found to elevate 560nm absorbance by 6%.

Trolox: Trolox was found to cause elevated estimation of LOOH when the recommended calibration procedure was not employed. However, when calibration as suggested, no significant differences were noted between plasma with and without Trolox.

No Internal Calibration		Internal Calibration	
Plasma	11.7	Plasma	16.1
Plasma + Trolox	17.6	Plasma + Trolox	14.8

Other: The following potential interferents are reported to have no effect on the XOF assay.[3]

Linoleic acid	(4 µM)
Glucose	(25 mM)
Fructose	(25 mM)
Galactose	(25 mM)
Glycerol	(25 mM)
Cholesterol	(100 µM)
Triglyceride	(100 µM)
GSH	(100 µM)
Vitamin E	(500 µM)
Lens crystalline	(100 µg/mL)
Bovine serum albumin	(100 µg/mL)

Storage Instructions:
Upon receipt, store the reagents at 2-8ºC. Unopened reagents are stable for 6 months from the date of manufacture. Do not use components beyond the expiration date printed on the kit box label.

Opened reagents, except for reconstituted BHT, must be stored at 2-8 ºC and should be used in a timeframe according to specific reagent guidelines.

Reconstituted BHT should be stored at <–20 ºC.

Assay Preparation:
Instrument Setup

Wavelength:	560 nm
Mode:	Endpoint

Reagent Preparation:
BHT Stock Reagent
Pour entire contents of the BHT solvent into the BHT Reagent bottle. Agitate until BHT Reagent is fully dissolved. Store on ice during use. Place at <–20 ºC if not used the same day.

Working MeOH:BHT Reagent
Calculate required volume as 0.5 mL x number of vials + 1 mL.
Add required volume of methanol to container (with closure).
Add 10 µL BHT Reagent per mL methanol to container. Mix by swirling.
Label as **"Working MeOH:BHT".** Store on ice and use the same day as preparation. Prior to use, bring to room temp.

Working XOF Reagent
Prepare and use just before use. Calculate required volume as 25 µL x number of vials + 250 µL. Combine *Xylenol Orange Reagent* and *Iron Reagent* in equal volumes (1:1) to generate required volume of XOF. Label as "**Working XOF Reagent".**

Sample Handling/Preparation:
General guidelines are provided below for various sample types.

Plasma
Heparinized plasma is suitable for use with this assay. EDTA plasma is not suitable for use and should be strictly avoided due to the ability of EDTA to render iron unavailable for reaction with Xylenol Orange.

Tissues
Liver and muscle sample have been tested successfully using this method. Tissue should be homogenized (10-20% w/v) in PBS. Later dilution can be performed if necessary.

Assay Protocol:
Standard Method:
1. Label 3 vials as: Calibrator (A), Reagent Blank (B) and Reduced Reagent Blank (C) *(Determine each of these assay constants only once for each run)* .

2. Label 3 vials for each unknown sample replicate: Sample (X), Sample Blank (Y) and Sample Recovery (Z).

3. **Add 100 µL water** to vials A, B and C.

4. Add **100 µL sample** to vials X, Y and Z.

5. Add 5 µL **Catalase Reagent** to all vials.

6. Vortex briefly (1 second) to mix.

7. Incubate 10 minutes at room temperature.

8. Add **500 µL Working MeOH:BHT Reagent** to all vials.

9. Cap and vortex 20-30 seconds (20 count).

10. Centrifuge at 10,000 Xg for 3-5 minutes.

11. Add **10 µL water** to vials B and X.

12. Add **10 µL TCEP Reagent** to vials C and Y.

13. Add **10 µL Calibrator** to vials A and Z.

14. Cap and Vortex briefly (1 second) to mix.

15. Incubate 1 hour at room temperature.

16. Add 50 **µL Working XOF Reagent** to all vials.

17. Cap and Vortex briefly (1 second) to mix.

18. Incubate 1 hour at room temperature.

19. Centrifuge at 10,000 Xg for 3-5 minutes.

20. Transfer supernatant to a cuvette and measure absorbance at 560 nm.

Data Analysis:

Analysis of sample data is based on the molar absorption coefficient of a given compound being equal to the measured absorbance divided by the concentration of the analyte multiplied by the effective path length (1cm):

$$\varepsilon_{560} = \frac{A_{560}}{[LOOH]}$$

Rearrangement yields:

$$[LOOH] = \frac{A_{560}}{\varepsilon_{560}} \quad \text{or} \quad \frac{A_X - A_Y - A_{B\text{-}C}}{\varepsilon_{560}}$$

Where ε_{560} for MeHPODE = 0.0337
A_X = Abs Sample (X)
A_Y = Abs Sample Blank (Y) (Sample + TCEP)
$A_{B\text{-}C}$ = Abs Reagent Blank (B) - Abs Reduced Reagent Blank (C)

Calibrator Concentration:

$$[\text{Calibrator}] = \frac{A_A - A_B}{\varepsilon_{560}}$$

Where ε_{560} for MeHPODE = 0.0337
A_A = Calibrator (A) Absorbance
A_B = Reagent Blank (B) Absorbance

To utilize the calibrator value, begin with the equation:

$$\frac{[\text{Sample LOOH}]}{[\text{Calibrator}]} = \frac{\text{Net Sample Absorbance}}{\text{Absorbance due to Spiked Calibrator}}$$

Rearrangement yields:

$$[\text{Sample LOOH}] = [\text{Calibrator}] \bullet \frac{A_X - A_Y - A_{B\text{-}C}}{A_Z - A_X}$$

Where A_X = Sample (X) Absorbance
A_Y = Sample Blank (Y) (Sample + TCEP) absorbance
A_Z = Calibrated Sample (Z) (Sample + Calibrator) absorbance
$A_{B\text{-}C}$ = Abs Reagent Blank (B) - Abs Reduced Reagent Blank (C)

Data Analysis (continued):

Example Data and Calculations:

Aged porcine plasma samples were tested in triplicate using the NWK-LHP method. The absorbance at 560 nm was recorded for each parameter and the data is shown below:

A_{560}	1	2	3	Avg
A_A	0.2392	0.2167	0.2323	0.2294
A_B	0.0471	0.0499	0.0470	0.0480
A_C	0.0464	0.0384	0.0435	0.0428
A_X	0.2149	0.2022	0.2023	0.2064
A_Y	0.0873	0.0743	0.0890	0.0835
A_Z	0.3168	0.3070	0.3218	0.3152

Calculate the value for the calibrator:

$$[\text{Calibrator}] = \frac{A_A - A_B}{\varepsilon_{560}} = \frac{0.2294 - 0.0480}{0.0337} = 5.38\ \mu M$$

$$[\text{Sample LOOH}] = [\text{Calibrator}] \bullet \frac{A_X - A_Y - A_{B\text{-}C}}{A_Z - A_X}$$

$$[\text{Sample LOOH}] = [\text{Calibrator}] \bullet \frac{A_X - A_Y - (A_B - A_C)}{A_Z - A_X}$$

$$[\text{Sample LOOH}] = 5.38\ \mu M \bullet \frac{0.2064 - 0.0835 - (.0480 - .0428)}{0.3152 - 0.2064}$$

[Sample LOOH] = 5.82 μM in reaction mix, 35.50 μM in original sample

Performance Characteristics:

Sensitivity:

Sensitivity is dependent on the ability of an instrument to differentiate between low level absorbances and individual sample matrix issues.

Estimated sensitivity = 0.3 μM in reaction mix
1.9 μM in original sample

Performance Characteristics (continued):

Linearity: on Dilution:

The dilutional response of varying amounts of LOOH added to the assay is linear across a wide range in both pure systems as well as complex biological systems such as plasma and tissue.

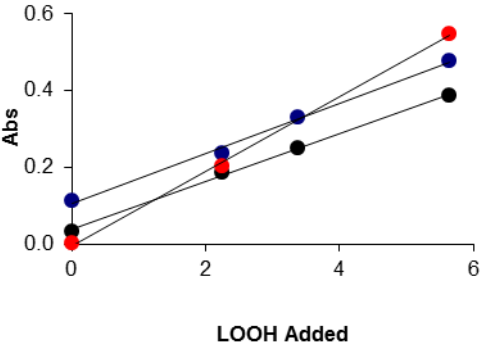


Figure 8:The absorbance of plasma (•), plasma less the absorbance from TCEP reduced plasma (•) and water (•) was measured at increasing concentrations of LOOH.

Accuracy:

Recovery of LOOH analyte is variable depending on sample type and can be greatly enhanced through the use of the internal calibrator (supplied).

Precision:

Intra Assay Replicate Precision = 4 %

References:

1. Nelson, D.L. and Cox M.M., *Lehninger Principals of Biochemistry*, 3rd ed., Worth Publishers, New York 2000.

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

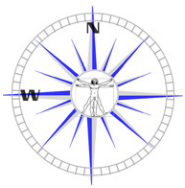
3. Jiang, Z-Y., et.al., (1991), *Analytical Biochemistry* 202, 384-389.

4. Nourooz-Zedeh, J., et.al., (1994), *Analytical Biochemistry* 220, 403-409.

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:



Northwest
Life Science Specialties, LLC

5131 NE 94th Avenue, Suite 201
Vancouver, WA 98662
Phone 360-449-3091 or Toll Free: 888-449-3091
Fax 360-449-3092
E-mail: sales@nwlifescience.com