OxisResearch™
A Division of AOXRE LLC

BIOXYTECH®

Lipid Peroxidation Microplate (LPO-MP™) Assay Kit

Catalog Number 21032
For Research Use Only.
Store at 2 to 8°C.

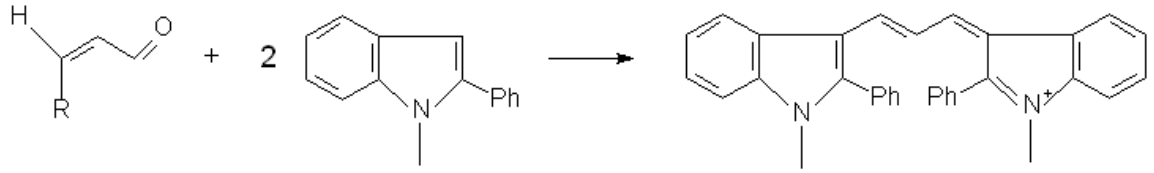
INTRODUCTION

The Analyte

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. Measurement of malondialdehyde and 4-hydroxyalkenals has been used as an indicator of lipid peroxidation (1). Lipid peroxidation products: malondialdehyde (MDA) and, in particular, 4-hydroxy-2-nonenal (4-HNE), are not only its physiological and protective function as signaling molecule stimulating gene expression and cell survival, but also its cytotoxic role inhibiting gene expression and promoting cell death (2). It is widely agreed that lipid peroxidation is closely related to many diseases (3). The method of lipid peroxidation assay kit is designed to test MDA and 4-hydroxyalkenals in methanesulfonic acid.

Principles of the Procedure

This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of reagent R1 to yield a stable chromophore with maximal absorbance at 586 nm. For simultaneous determination of MDA and 4-hydroxyalkenals, one must use the procedure utilizing methanesulfonic acid (MSA) as the acid solvent. The procedure in which HCl is used will only detect MDA, since the 4-hydroxyalkenals do not form a chromophore with reagent R1 under those conditions.



MDA : R = OH
4-hydroxyalkenal : R= hydroxyalkyl

$\lambda_{max} = 586_{max}$ nm

REAGENTS

Materials Provided (for 96 tests)

- Reagent R1 — N-methyl-2-phenylindole in acetonitrile, 1 X 13 mL
- Reagent R2 — Methanesulfonic acid (MSA), 1 X 5 mL
- MDA Standard — 1,1,3,3-Tetramethoxypropane (TMOP) in Tris-HCl, 1 X 200 µL
- Diluent — Ferric Iron in Methanol, 1 X 5.5 mL
- Microplate — 96-well Microplate, 1 X plate

Materials Required But Not Provided

- Spectrophotometer plate reader with a 586 nm filter (filters between 580 nm or 590 nm are also acceptable).
- Water bath, set to control temperature at 45 ± 1°C.
- Disposable glass test tubes and stoppers compatible with acetonitrile, methanol, and acid.
- Butylated hydroxytoluene (BHT)
- Acetonitrile
- Microcentrifuge
- Acid/acetonitrile resistant (e.g., polypropylene) microcentrifuge tubes.

Warnings and Precautions

- Do not smoke, eat or drink in areas where samples and reagents are handled.
- Wear disposable gloves when handling samples and reagents.
- Do not pipette reagents or samples by mouth.
- In case of accidental exposure of skin, mucous membranes or eyes to **R1** or **R2** reagents, thoroughly wash the exposed area with water.
- For *in vitro* use only. For research purposes only. Not for use in diagnostic procedures.

Reagent Storage and Handling

- Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
- It is good practice to transfer the desired volume of reagents for an experiment to a clean glass test tube or other vessel and return the stock reagent bottles to 4°C.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 4°C. Reagent R2, methanesulfonic acid (MSA), freezes at 19°C or colder. This reagent does not need to be refrigerated, but, if it is stored with the rest of the assay kit at 2-8°C, it may easily be thawed by leaving at room temperature for a few hours prior to use.
- New pipettes or pipette tips should be used when removing reagents from the stock bottle. Avoid contaminating the stock bottles as this could affect assay performance.
- If reagents are handled and stored properly as described above, they are stable until the indicated expiration date.

PROCEDURE

Sample Preparation

Note: Please read the appropriate **NOTES** sections before starting sample preparation procedure.

Preservative To Prevent Sample Oxidation

AOXRE recommends that the researcher add BHT to a final concentration of 5 mM in the buffer prior to homogenization of tissue or cells. BHT can be made as a 100X stock 0.5 M solution in acetonitrile. If no antioxidant is added, new lipid peroxidation can occur during homogenization and biased values will result (4).

Concentration Of Tissue Homogenate/Sensitivity

Sample homogenates should be made as concentrated as possible. The concentration of protein in the homogenate should be determined. It is recommended that 0.2 mL of a homogenate containing 15-60 mg/mL protein be assayed for initial studies in a previously untested biological sample. For tissue culture cells, it is recommended that a sample derived from 10^7 cells be added to the LPO-586 assay (i.e., 0.2 mL of 1 X 10^7 per mL).

Sample Stability

Unless assayed immediately, samples should be frozen at -70°C to prevent loss of MDA and 4-hydroxyalkenals (HAE) (5,6) and prevent new sample oxidation. Samples should not be stored at -20°C. Once thawed from -70°C storage for assay, the sample should not be refrozen.

Samples should be protected from light to avoid photoxidation.

Sample preparation procedure

Tissue Homogenates (9,10)

1. If necessary, remove blood, *in situ* by perfusion or *in vitro* by rinsing, with ice-cold isotonic saline (i.e., 0.9% NaCl)
2. Weigh Tissue. Researchers must determine the optimal amount of tissue to use for their particular application, but a reasonable starting point would be 1gm tissue per 10ml of buffer .
3. Prepare tissue homogenate. Appropriate buffers would be ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.4) or ice-cold Tris buffer (20 mM, pH 7.4). Other buffers may be used, but the researcher should confirm non-interference in the assay by comparing the performance of the TMOP standard diluted in the chosen buffer with the standard in PBS.
4. Prior to homogenization, 10 µL 0.5 M BHT in acetonitrile should be added per 1 ml of tissue homogenate. This is to prevent sample oxidation during homogenization. A precipitate is expected. This precipitate will be removed by centifugation and will not affect the outcome of the assay.
5. After homogenization, centrifuge the homogenate. 3000 g at 4°C for 10 minutes should be sufficient, but more may be required if this does not remove all the turbidity from the supernatant.
6. The clear supernatant is used for the assay. Aliquots of this should taken for sample replicates, as well as for protein determination. 200 uL portions are required for use in this assay.
7. The supernatant should be kept on ice prior to analysis, or, if not analyzed immediately after preparation, frozen at –70°C for longer storage.

Cell Culture (11,12)

1. Remove cells using rubber policeman. Lysis buffers have a high potential of interfering in this assay. Cells should be washed well in ice-cold PBS or Tris buffer (20 mM, pH 7.4), and resuspended in the same buffer. Researchers should determine the optimal number of cells to use in this assay, but a recommended starting point is 5×10^7 cells per ml.
2. AOXRE recommends cell lysis by sonication, but other researchers have used homogenization or freeze-thaw cycles. To prevent sample oxidation during preparation, lysis should be done in the presence of 10 uL 0.5 M BHT per 1 ml of cell homogenate.
3. After homogenization, follow steps 5-7 of tissue homogenization procedure.

Cells cultured in serum containing medium should be washed several times to remove serum components prior to homogenization.

Plasma or Serum

The amount of free MDA or HAE in normal plasma or serum is at or below the limit of detection of the Lipid Peroxidation (LPO-586) assay.

Reagent Preparation

Dilution of the R1 solution for use in the assay. Dilute ratio: R1 3:1 with Diluent (i.e. 12 mL R1 + 4 mL Diluent). Prepare this solution immediately before use. Do not leave the R1 reagent bottle uncapped (open to the atmosphere).

Preparation of Standards

Malondialdehyde is provided as an acetal because the aldehyde itself is not stable. The acetal (TMOP) is hydrolyzed during the acid incubation step at 45°C, which will generate MDA.

The TMOP standard is provided as a 10 mM stock solution which will need to be diluted 1/500 (v/v) in deionized water just prior to use to yield a 20 µM stock solution for use in the assay. For a standard curve, pipette the volumes shown in table 1 to give a total of 200 µL of each standard.

Use the zero concentration standard(A_0) as a blank to zero the spectrophotometer or plate reader that the assay is to be run on. If it is easier, one may also subtract the absorbance value of the blank from the absorbance value of each of the standards, as described in the calculations section.

Table 1: Standard Curve Dilution Volumes

Standard	MDA Conc. (µM)	Volume of dH_2O (µL)	Volume of 20 µM MDA Stock sol. (µL)
S_0	0	200	-
S_1	0.5	195	5
S_2	1.0	190	10
S_3	2.5	175	25
S_4	5.0	150	50
S_5	10.0	100	100
S_6	15.0	50	150
S_7	20.0	-	200

Sample Blank (Asb)

75% Acetonitrile / 25% Diluent. Add 650 µL to a microcentrifuge tube. Skip assay steps 1 and 2. The acid addition and the incubation steps are carried out on this blank.

Aaasy Procedure for Lipid Peroxidation (MDA and HAE)

In running an assay for the sum of MDA + HAE, AOXRE recommends the use of MDA as the standard. Most studies that report concentrations of free MDA and HAE indicate an approximate 10-fold excess of MDA. Since the slopes of the calibration curves for MDA and HNE are not identical, some error is unavoidable in the determination of the two compounds together. However, the use of an MDA standard curve will minimize the error involved.

(1). Add 140 µL of Standards or Samples to a microcentrifuge tube.
(2). Add 455 µL of diluted Reagent R1 to each tube and vortex.
(3). Add 105 µL R2 Reagent to each tube and mix well.
(4). Incubate at 45°C for 60 minutes.
(5). Centrifuge samples at 15,000 x *g* for 10 minutes to obtain a clear supernatant.
(6). Transfer the 3 x 150 µL of the supernatant to the microplate and read at 586 nm. See Scheme I for a sample plate layout. The color is stable for at least an hour at room temperature, or 2 hours at 4°C when stored in the dark.

Note: It is also acceptable to read a wavelength at between 580 nm and 590 nm.

Scheme I: Sample Plate Layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	S0	S0	S0	BLK	BLK	BLK	U8	U8	U8	U16	U16	U16
B	S1	S1	S1	U1	U1	U1	U9	U9	U9	U17	U17	U17
C	S2	S2	S2	U2	U2	U2	U10	U10	U10	U18	U18	U18
D	S3	S3	S3	U3	U3	U3	U11	U11	U11	U19	U19	U19
E	S4	S4	S4	U4	U4	U4	U12	U12	U12	U20	U20	U20
F	S5	S5	S5	U5	U5	U5	U13	U13	U13	U21	U21	U21
G	S6	S6	S6	U6	U6	U6	U14	U14	U14	U22	U22	U22

H	S7	S7	S7	U7	U7	U7	U15	U15	U15	U23	U23	U23

Calculations

1. Using the standard data, calculate the net A_{586} for each standard by subtracting the blank (A_o) value from each of the standard A_{586} values. Plot net A_{586} vs [MDA], and perform a linear regression analysis of A_{586} on [MDA]:

$$[A_{586}] = a[MDA] + b$$

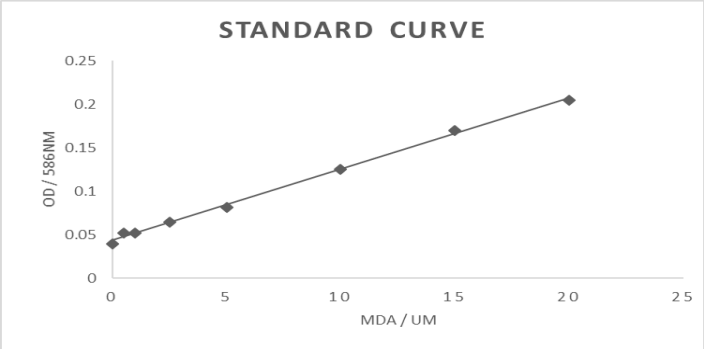
2. Calculate the concentration of analyte in each unknown from the net A_{586} of the sample (If a sample blank was required, subtract A_{sb} from the net sample absorbance.):

$$[MDA] = \frac{A_{586} - b}{a} \bullet df$$

Where:

[MDA] is the µM concentration of MDA in the sample
A_{586} = Net absorbance at 586 nm of the sample
a = regression coefficient (slope)
b = intercept
df = dilution factor

Figure 1: Example of MDA Standard Curve



LIMITATIONS

1. Sucrose or fructose at concentrations of ≥50 mM in the sample will cause a high bias in the assay. Vitamin E, when present at ≥15 µM, can cause a diminution in the values obtained for 4-HNE.
2. Although the standards in this assay will usually appear blue, the samples or blanks sometimes appear another color, such as pink or green. This is due to chromophores that form other than those producing the 586 nm peak.
Ordinarily, these chromophores will not interfere with the A586.
3. This assay measures only free MDA or 4-HNE in samples. The conditions of the assay do not provide for liberation of MDA bound to proteins via Schiff Base(7). 4-HNE is sufficiently reactive that it rapidly combines with proteins in tissues, forming stable adducts that are not liberated by heating at high temperatures in acid; as a consequence, there is very little free 4-HNE in tissue (1).
4. Normal tissues have very low levels of free MDA or 4-HNE, typically 10-100 pmol/mg protein (4,8). Assay of a 0.2 mL sample containing 10 mg of protein derived from normal tissue will give absorbance values at 586 nm of 0.01 or less in this assay. Caution must be taken not to interpret

very low absorbance values as an accurate reflection of analyte concentrations in biological samples.
5. In setting up this assay for the first time on a particular biological sample, the kinetics of color development on the sample should be followed in comparison with that of the TMOP standard. The A586 of the sample should reach a plateau and then remain stable. If the A586 continues to go up after the standards have achieved a stable color, the researcher should be concerned that non-MDA reactivity (interference) is occurring in the sample.
6. In setting up this assay for the first time on a particular biological sample, a wavelength scan from 450 to 700 nm should be performed on the clarified sample reaction mixture and compared to that obtained with the TMOP standard. The lack of a peak at 586 nm or lack of reasonable definition to the sample profile compared to the standard would suggest interference in the sample.
7. If no antioxidant is added to the samples during homogenization and subsequent assay, a high bias due to new sample oxidation may result.

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*Oxis*Research™

1499 Rollins Road

Burlingame, CA 94010 U.S.A.
E-mail: info@aoxre.com
Telephone: 650-289-8908
www.aoxre.com

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