*Oxis*Research™

A Division of AOXRE LLC

BIOXYTECH® LPO-586™

Colorimetric Assay For Lipid Peroxidation
For Research Use Only. Not For Use In Diagnostic Procedures.
Catalog Number 21012

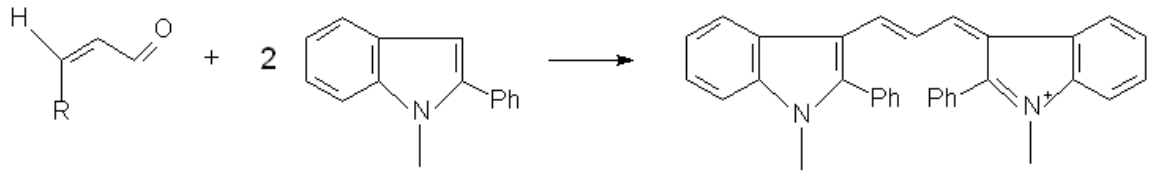
INTRODUCTION

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). The LPO-586 method is designed to assay either MDA alone (in hydrochloric acid) or MDA in combination with 4-hydroxyalkenals (in methanesulfonic acid.)

Principles of the Procedure

The LPO-586™ 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 100 tests)

- Reagent R1 — N-methyl-2-phenylindole in acetonitrile, 3 X 18 mL
- Reagent R2 — Methanesulfonic acid (MSA), 1 X 16.5 mL
- MDA Standard — 1,1,3,3-Tetramethoxypropane (TMOP) in Tris-HCl, 1 X 1 mL
- Diluent — Ferric Iron in Methanol, 1 x 30 mL

Materials Required But Not Provided

- Spectrophotometer for measuring absorbance at 586 nm from 0-2 absorbance units.
- Spectrophotometric cuvettes with a 1 cm optical path length.
- Water bath, set to control temperature at 45 ± 1°C.
- Disposable glass test tubes and stoppers compatible with acetonitrile, methanol, and acid.
- HCl, 37%
- 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

Reagent Preparation

Dilution of the R1 solution for use in the assay. Add one volume (6 mL) of Diluent to three volumes (18 mL) reagent R1. Prepare this solution immediately before use. Do not leave the R1 reagent bottle uncapped (open to the atmosphere).

37% HCl Preparation

37% HCl is approximately 12 N acid reagent. This is the strength of concentrated HCl available from many chemical companies. **DO NOT DILUTE THE HCl PRIOR TO USE IN THE ASSAY.**

Sample Preparation

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

Preservative To Prevent Sample Oxidation

OXIS 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 (2).

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) (3,4) 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 (7,8)

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 uL 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^{o}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^{o}C for longer storage.

Cell Culture (9,10)

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 x 10^{7} cells per ml.
2. OXIS 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 LPO-586 assay.

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 on page 5.

Table 1: Standard Curve Dilution Volumes

Target concentration of standard in reaction mixture, µM	**0**	**0.50**	**1.00**	**2.00**	**3.00**	**4.00**
Volume of 20 µM standard to add (mL)	0	25	50	100	150	200
Volume of diH_2O or buffer used for diluting or mixing sample to add (mL)	200	175	150	100	50	0

ASSAY

Assay Procedure for MDA (Hydrochloric Acid Solvent Procedure)

1. Prepare the standards according to Table 1 in clean glass test tubes or polypropylene microcentrifuge tubes. Preferably, standards should be run in triplicate.
2. Add 200 µL of sample to a clean glass test tube or polypropylene microcentrifuge tube. Unknowns should be run in triplicate.
3. Add 650 µL of diluted R1 reagent.
4. Mix gently by vortexing the sample.
5. Add 150 µL concentrated (12 N) HCl.
6. Mix well and stopper the tube.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 15,000 x *g* for 10 minutes) to obtain a clear supernatant.
9. Transfer the clear supernatant to a cuvette.
10. Measure the absorbance at 586 nm.*

Assay Procedure for MDA + HAE (MSA Solvent Procedure)

In running an assay for the sum of MDA + HAE, OXIS 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. Prepare standards according to Table 1 in clean glass test tubes or polypropylene microcentrifuge tubes. Preferably, standards should be run in triplicate.
2. Add 200 μL of sample to a clean glass test tube or polypropylene microcentrifuge tube. Unknowns should be run in triplicate.
3. Add 650 μL of diluted R1 reagent.
4. Mix gently by vortexing the sample.
5. Add 150 μL R2 reagent.
6. Mix well and stopper the tube.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 15,000 x *g* for 10 minutes) to obtain a clear supernatant.
9. Transfer the clear supernatant to a cuvette.
10. Measure absorbance at 586 nm.*

*The color is stable for at least an hour at room temperature, or 2 hr at 4°C if the samples are stored in the dark and no evaporation occurs.

Sample Blank (Asb)

A sample blank should be measured to correct for any A_{586} contribution due to the sample. This blank is made by adding 650 μL of 75% acetonitrile/25% Diluent instead of the diluted R1 reagent to the assay tube. The acid addition and sample incubation steps are then carried out as described above.

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.):

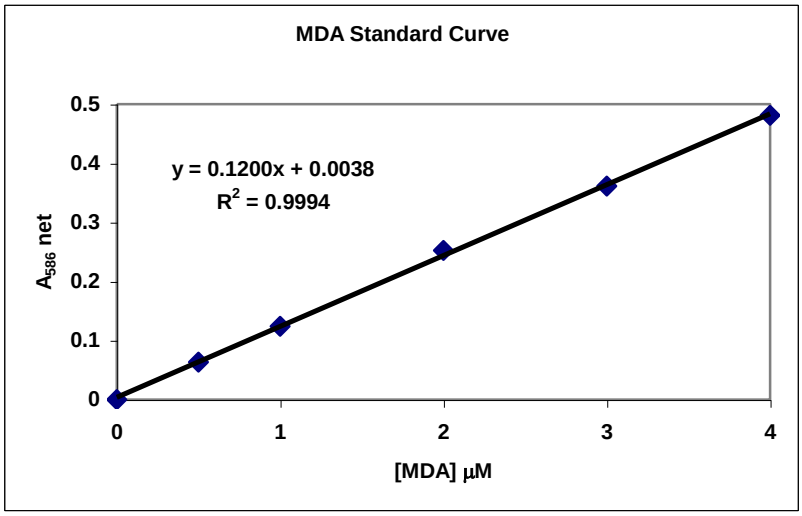
$$[\mathrm{MDA}] = \frac{A_{586} - b}{a} \bullet \mathrm{df}$$

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



PERFORMANCE CHARACTERISTICS

Least Detectable Concentration

Experiments on enaldehyde standards and blanks suggest that the analytical limit of detection in a purified system will be 0.1 nmol/mL final concentration (0.5 nmol/mL in the sample), corresponding to an absorbance value of approximately 0.011. The limit for biological samples will generally be higher and it is recommended that the researcher assess this parameter in their system.

Reproducibility

Experiments in which standard samples (0-20 µM) were assayed using the same protocol over a period of 10 days established the standard error of the measurement (SEM) at less than 5%.

LPO-586 Assay in microtiter plates

For those who are interested in the application of the LPO-586 assay in microtiter plates, we provide the following information.

1. OXIS has not validated the use of the LPO-586 assay in microtiter plates.
2. The 45°C incubation and sample clarification (e.g., centrifugation) MUST be performed in stoppered test tubes/microcentrifuge tubes to ensure adequate mixing, a minimum loss of solvent due to evaporation, and successful sample clarification.
3. Measuring the absorbance of clarified samples in microtiter plates:
   a. The extinction coefficient for MDA/4-HNE will be different according to the effective path length in the particular microtiter plate reader.
   b. The absolute absorbance as a function of enaldehyde concentration will be lower, decreasing the assay sensitivity.
   c. If continuous wavelength monitoring is not available, 580 nm or 590 nm filters should be obtained to measure the samples.

d. The user must establish that the particular microtiter plate to be used is chemically resistant to acetonitrile and strong acid.

4. Volume of the reaction mixture: A minimum of 0.5 mL total reaction mixture should be used to assure 0.2-0.3 mL of clarified sample be available for determining absorbance.
5. Once the conditions for measuring the absorbance of clarified samples in microtiter plates are established, they must be performed the same way each time.

NOTES

Limitations

1. Sucrose or fructose at concentrations of 50 mM or greater in the sample will cause a high bias in the assay. Vitamin E, when present at 15 µM or greater, can cause a diminution in the values obtained for the 4-hydroxyalkenals.
2. Although the standards in this assay will usually appear blue, at the time of analysis, samples or blanks can 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 absorbance at 586 nm.
3. The LPO-586 assay measures only free malondialdehyde or 4-HNE in samples. The conditions of the assay do not provide for liberation of MDA bound to proteins via Schiff Base (5). 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 malondialdehyde or 4-HNE, typically 10-100 pmol/mg protein (2,6). 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 the LPO-586 assay. Caution must be taken not to interpret very low absorbance values (near zero) as an accurate reflection of analyte concentrations in biological samples.
5. In setting up the LPO-586 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 A_{586} of the sample should reach a plateau and then remain stable. If the A_{586} 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 the LPO-586 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 or non-specific reactivity 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.
8. If turbid or hazy reaction mixtures do not clarify upon centrifugation, further efforts should be made to purify the sample. Turbid samples will give biased values in the assay.

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REFERENCES

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