_Oxis_Research™
LumiQuick Diagnostics, Inc.

BIOXYTECH®

High Sensitivity Malondialdehyde Microplate (HS MDA-MP™) Assay Kit

Catalog Number A21033 (AOXRE Catalog Number 21033)
For Research Use Only.
Store at 2 to 8°C.

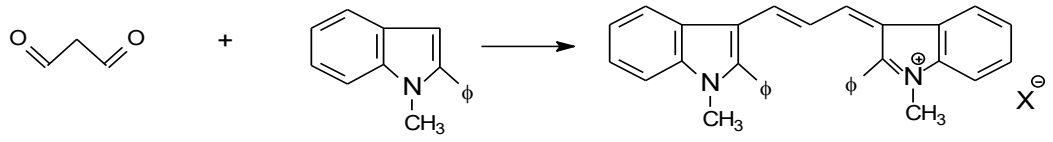
INTRODUCTION

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, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. Malondialdehyde (MDA) is the most frequently used biomarker of oxidative stress in many health problems such as cancer, psychiatry, chronic obstructive pulmonary disease, asthma, or cardiovascular diseases (1, 2). Therefore, measurement of malondialdehyde is widely used as an indicator of lipid peroxidation (3). Increased levels of lipid peroxidation products have been associated with a variety of chronic diseases in both humans (4, 5) and model systems (6, 7). MDA reacts readily with amino groups on proteins and other biomolecules to form a variety of adducts (3), including cross-linked products (8). MDA also forms adducts with DNA bases that are mutagenic (9, 10) and possibly carcinogenic (11). DNA-protein cross-links are another result of the reaction between DNA and MDA (12). The TBARS method is commonly used to measure MDA in biological samples (13). However, this reaction is relatively nonspecific; both free and protein-bound MDA can react. The method of high sensitive malondialdehyde assay kit is designed to assay free MDA or, after a hydrolysis step, total MDA (i.e., free and protein-bound Schiff base conjugates). The assay conditions serve to minimize interference from other lipid peroxidation products, such as 4-hydroxyalkenals.

PRINCIPLES OF THE PROCEDURE

The MDA-586 method [1] (14) is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1, NMPI), with MDA at 45°C. One molecule of MDA reacts with 2 molecules of NMPI to yield a stable carbocyanine dye as shown in **Figure 1** (15).



Malondialdehyde (MDA) | NMPI | Carbocyanine Dye

Figure 1. N-methyl-2-phenylindole (NMPI) reacts with malondialdehyde to form an intensely colored carbocyanine dye with a maximum absorption at 586 nm.

[1] US Patent No. 5726063

The MDA-586 method is specific for MDA because 4-hydroxyalkenals do not produce significant color at 586 nm under the conditions of the assay (16). **Figure 2** shows the absorption spectra of the reaction products of MDA under the standard MDA-586 reaction conditions. The reaction is carried out in hydrochloric acid (provided) and with the addition of Probucol (provided), an antioxidant, to further minimize the reaction of 4-hydroxyalkenals. Under these conditions, there is little absorbance at 586 nm from HNE, the most common 4-hydroxyalkenal produced in cells subjected to lipid peroxidation.

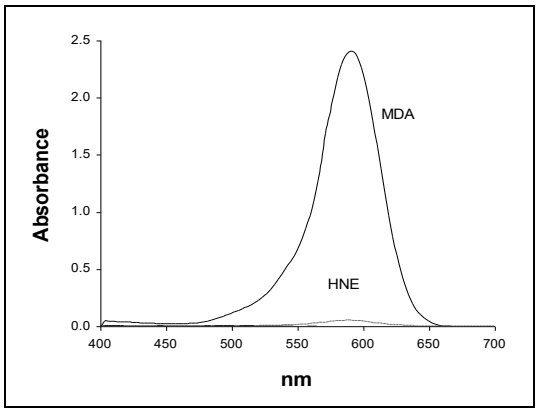


Figure 2. Absorption spectra obtained from the reaction of NMPI with MDA (21 µM) or HNE (19 µM) in the presence of HCl.

In the MDA-586 assay, a calibration curve is prepared using the MDA standard provided. The [MDA] in an unknown and is determined from the absorbance of the unknown at 586 nm in the MDA-586 assay and the standard curve. For colored samples, a sample blank is run (omitting the NMPI) and any absorbance at 586 nm is subtracted from the sample absorbance to give the true absorbance due to the carbocyanine dye.

REAGENTS

Materials Provided (for 96 tests)

- Reagent R1 — N-methyl-2-phenylindole, in acetonitrile, 1 X 13 mL
- Reagent R2 — Concentrated hydrochloric acid, 1 X 5 mL
- MDA Standard — 1,1,3,3-tetramethoxypropane (TMOP) in Tris-HCl, 1 X 200 µL
- BHT — BHT (butylated hydroxytoluene) in acetonitrile, 1 X 2 mL
- Probucol — Probucol in methanol, 1 X 1 mL
- 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 or heat block set to control temperature at 45 ± 1°C
- Disposable tubes and stoppers (glass or polypropylene)
- Microcentrifuge

Warnings and Precautions

- **Acetonitrile** (R1 and BHT) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Causes irritation. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Hydrochloric acid** is corrosive and may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **Methanol** (Probucol) is a flammable liquid and is harmful if swallowed, inhaled, or absorbed through the skin. Use with adequate ventilation. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.
- **BHT** is harmful if swallowed, inhaled, or absorbed through the skin. Risk of serious eye injury. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
- **Probucol** is harmful if swallowed, inhaled, or absorbed through the skin. In case of contact with skin or eyes, rinse immediately with plenty of water and seek medical advice.

Reagent Storage and Handling

- 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 storage.
- 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.
- Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Sample Preparation

- Sample Oxidation. AOXRE recommends that butylated hydroxytoluene (BHT) be added to a final concentration of 5 mM prior to homogenization of tissue or cells. BHT is provided as a 500 mM solution in acetonitrile. If no antioxidant is added, new lipid peroxidation can occur during homogenization, resulting in biased values (13).
- Total MDA**.** The standard MDA-586 method is designed to measure free MDA. Determination of total MDA requires hydrolysis of the sample in the presence of BHT, at pH 1-2 at 60°C for 80 minutes. The hydrolysis yield is markedly reduced at pH less than 1 and pH greater than 2 (15).
- Tissue. Sample homogenates should be as concentrated as possible, (i.e., approximately 20% - 30% or 200-300 mg tissue per mL of buffer). It is recommended that 0.2 mL of a homogenate containing 15-60 mg/mL of protein, should be assayed for initial studies of a previously untested biological sample. Homogenates not assayed immediately must be stored at –70°C or lower (19, 20).
- Cell culture. Cells should be washed to removed protein and other constituents from the media, then lysed by 2 or 3 freeze/thaw cycles or by sonication. Cell debris are then removed by centrifugation at 3000 x *g* for 10 minutes at 4°C. It is recommended that a lysate from 10^7 cells be added to the MDA-586 reaction mixture (i.e., 0.2 mL of 5 X 10^7 cells per mL). Lysates not assayed immediately must be stored at –70°C or lower (19, 20).
- Plasma. The concentration of free MDA in normal plasma is below the limit of quantitation of the MDA-586 method (18). Bound MDA, which represents over 80% of the plasma MDA (19), can be hydrolyzed in the presence of BHT, at pH 1-2 at 60°C for 80 minutes. The hydrolysis yield is markedly reduced at pH less than 1 and pH greater than 2 (15). See below for protocol. Because MDA has a room temperature half-life in plasma of approximately two hours, samples must be kept cold and assayed as soon as possible after hydrolysis (3). Plasma not assayed immediately must be stored at –70°C or lower (19, 20).

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 on.

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.

Assay

1. Add 7 µL of probucol to each assay tube.+
2. Add 140 µL of Standards or Samples to a microcentrifuge tube.
3. Add 448 µL of diluted Reagent R1 to each tube and vortex.
4. Mix by briefly vortexing each tube.
5. Add 105 µL of R2.
6. Stopper each tube and mix well by vortexing.
7. Incubate at 45°C for 60 minutes.
8. Centrifuge turbid samples (e.g., 10,000 X g for 10 minutes) to obtain a clear supernatant.
9. 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.
10. Measure absorbance at 586 nm.* (Also acceptable at between 580 nm and 590 nm).

+ Some of the probucol may precipitate but this will not cause a problem. Any precipitate formed is removed in Step 8.

*The color is stable for at least two hours at room temperature (15).

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]:

$$\left[\mathrm{A}_{586}\right] = a\left[MDA\right] + b$$

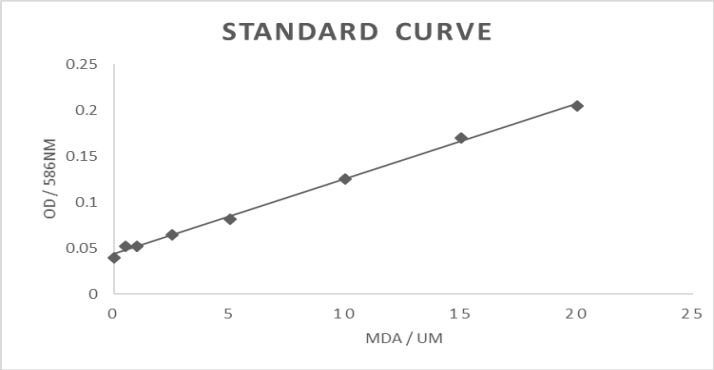
2. Calculate the concentration of analyte in each unknown from the net A_{586} of the sample (If a sample blank was required, subtract A_{sb} from the net sample absorbance.):

$$\left[\mathrm{MDA}\right] = \frac{\mathrm{A}_{586} - \mathrm{b}}{\mathrm{a}} \bullet \mathrm{df}$$

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



NOTES

Interference

- Reducing Sugars. Sucrose or fructose, at concentrations of 50 mM or greater in the sample, will cause a high bias in the assay (18).
- Antioxidants. Glutathione (200 µM), ascorbate (100 µM), probucol (1 mM), and BHT do not interfere. (15).
- Others. Hydrogen peroxide at 50 µM will reduce MDA by 13% (15).

Limitations

- Normal Plasma. MDA is at the detection limit in normal plasma.
- Normal Tissue. Normal tissues have very low levels of free malondialdehyde, typically 10-100 pmol/mg protein (13, 21). Assuming the lower value, an assay of a 0.2 mL sample containing 10 mg of protein derived from normal tissue will give an absorbance value at 586 nm of 0.012 in the MDA-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.

Assay Performance

- Sample *O*xidation. The kinetics of color development in the sample should be followed in comparison with that of the TMOP supplied with the reagent set. The A_{586} of the sample should reach a plateau and then remain stable. Continual increase in the A_{586} indicates non-MDA reactivity (interference) or oxidation is occurring in the reaction mixture. This could also be due to slow hydrolysis of protein-bound MDA.
- Turbidity. A wavelength scan from 400 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 a continuous increase in the baseline would suggest interference or non-specific reactivity in the sample (18).

Reagent Preparation

- R1 Dilution. Failure to dilute the R1 reagent with methanol will result in the formation of a 2 phase reaction mixture.
- Assay Buffers. Buffers should not contain amino groups (e.g., Tris) since these can react with MDA to form Schiff bases, which may hydrolyze only slowly under the MDA-586 reaction conditions. Potential interference in the assay should be assessed by adding MDA (hydrolyzed TMOP) to aliquots of buffer (water as a control) and assaying for free MDA after an appropriate incubation period, which is determined by the experimenter's own protocol for tissue preparation. A suggested protocol follows:

  1. Prepare 0.2 M HCl solution by mixing 17 μL of R2 with 983 μL of water.
  2. Combine 50 μL MDA Standard and 50 μL of 0.2 M HCl. Stopper, vortex, and allow it to incubate at room temperature for two hours. This will hydrolyze the TMOP, forming 5 mM MDA.
  3. Dilute 20 μL of the MDA to a final volume of 10 mL with the buffer being tested ([MDA] = 10 μM)
  4. Prepare an identical sample using water instead of buffer.
  5. Allow both samples to incubate at room temperature. Remove 200 μL aliquots at appropriate times, determined by the experimenter's own sample preparation protocol, and analyze using the MDA-586 assay. Phosphate buffers do not react with MDA and are recommended for sample preparation.

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