Colorimetric Microplate Assay for
Lipid Peroxidation
Product No. FR 22

For Research Use Only

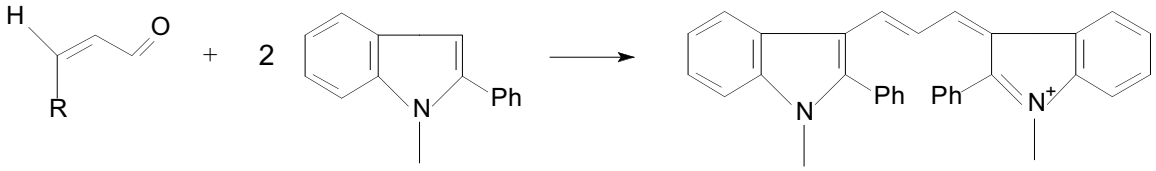
WARNING: This kit contains substances or reagents that may be harmful if used improperly. See MSDS for more information.

INTRODUCTION

Lipid peroxidation, a well-established mechanism of cellular injury in plants and animals, 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, and the measurement of MDA and HAE has been used as an indicator of lipid peroxidation (1). This method is designed to assay either MDA alone (in hydrochloric acid) or MDA in combination with HAE (in methanesulfonic acid.)

PRINCIPLES OF 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.



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

Max = 586 nm

MATERIALS PROVIDED (for 100 tests)

1.	Reagent R1	N-methyl-2-phenylindole in Acetonitrile, 1 x 13 mL
2.	Reagent R2	Methanesulfonic acid (MSA), 2 x 2.2 mL ***Caustic Acid***
3.	MDA Standard	1,1,3,3-Tetramethoxypropane in Tris-HCl, 1 x 200 μL
4.	Diluent	Ferric Chloride Hexahydrate in Methanol, 2 x 2.6 mL
5.	96 well microplate	12 strips x 8 wells in a frame
6.	Microplate Template	
7.	Kit Instruction	

MATERIALS NEEDED BUT NOT PROVIDED

1. Water bath or heat block set at 45°C
2. 12N HCl, 37% (v/v)
3. Butylated hydroxytoluene (BHT)
4. Acetonitrile

5. Microcentrifuge
6. Microcentrifuge tubes (100 x 1.5 mL polypropylene tube or equivalent)
7. Microplate reader capable of measuring absorbance at 586 nm (a 580 nm or 590 nm filter will also provide acceptable results)

STORAGE

Store this kit and its components at 4°C.
For information about partial kit storage, please see Procedural Notes #2.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as it may be detrimental to the assay.
3. Wear proper protective eyewear and gloves when handling samples and reagents.
4. Do not pipette reagents or samples by mouth.
5. In case of accidental exposure of skin, mucous membranes or eyes to **R1** or **R2** reagents, thoroughly wash the exposed area with water and seek immediate medical attention.

PROCEDURAL NOTES

1. Reagents can be used immediately upon removal from refrigeration.
2. When performing this kit in part, please adhere to the following:
   -All unused components should be returned to storage at 4 °C.
   -Unused portions of the microplate should be returned to the bag provided.
   -Create a standard curve for each performance of the assay.
3. Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
4. Do not allow the capped reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles on ice or at 4°C.
5. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.
6. If reagents are handled and stored properly as described above, they are stable until the indicated expiration date.

SAMPLE COLLECTION AND STORAGE

Unless assayed immediately, samples should be frozen at –70°C to prevent loss of MDA and (HAE) from *ex vivo* oxidation. Once thawed, samples should not be refrozen, and they should be protected from light to avoid photoxidation.

SAMPLE PREPARATION

To prevent sample oxidation, it is recommended that BHT be added to a final concentration of 5 mM in the buffer prior to homogenization as instructed in Step 2 of the homogenization procedure below. BHT can be made as a 100x (500 mM) stock solution in acetonitrile. If no antioxidant is added, *ex vivo* lipid peroxidation can occur during homogenization and elevated values will result.

Sample homogenates should be prepared 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 15-69 mg/mL protein be assayed for initial studies in a previously untested biological sample. For tissue culture cells, it is recommended that at least 1 x 10^7 cells per mL is used.

PLASMA OR SERUM
The amount of free MDA or HAE in normal plasma or serum is at or below the limit of detection of this assay.

CELL CULTURE AND TISSUE PREPARATION
Cell culture and tissue culture samples may require washing prior to homogenization.
Cells cultured in medium containing serum should be washed several times to remove serum components prior to homogenization. It is recommended that a homogenate of approximately 5 x 10^7 cells per mL be prepared for use in the assay. Cells can be washed in ice-cold isotonic saline (i.e., 0.9% NaCl). The cells are now ready for the homogenization procedure below.
Tissues may require the removal of blood by perfusion *in situ* with isotonic saline or *in vitro* by rinsing with ice-cold isotonic saline and the perfusate discarded. After washing, the tissue should be weighed prior to the homogenization procedure below.

HOMOGENIZATION PROCEDURE
1. Prepare homogenates in 20 mM phosphate buffer, pH 7.4. Other buffers may be used, but the researcher should confirm non-interference in the assay by measuring the MDA and/or 4-HNE standards diluted in the chosen buffer.
2. Prior to homogenization, add 10 μL 0.5 M BHT in acetonitrile to 1 mL of sample to prevent sample oxidation. A precipitate is expected. It will be removed by centrifugation and will not affect the outcome of the assay.
3. Centrifuge the homogenate to remove large particles (e.g., 3000 x *g* at 4°C for 10 minutes).
4. Remove an aliquot of the sample for protein determination.
5. Freeze the sample immediately at -70°C or keep on ice prior to testing. If desired, aliquot the samples to provide 0.2 mL of the homogenate for each assay.

REAGENT PREPARATION

Make the diluted R1 solution for the assay by adding one volume of Diluent to three volumes of Reagent R1. Prepare immediately before use. Do not leave the Reagent R1 bottle uncapped and open to the atmosphere.

STANDARD CURVE PREPARATION

Malondialdehyde is provided as an acetal because the aldehyde is not stable. The acetal (TMOP) is hydrolyzed during the acid incubation step, which generates MDA.

Note: The table below shows how to create an eight point standard curve using 24 wells. Fewer concentrations may be used at the discretion of the investigator.

The TMOP standard is provided as a 10 mM stock solution. Dilute the 10 mM stock TMOP 1:500 (v/v) in water just prior to use yielding a 20 μM stock solution. For a standard curve, combine the volumes shown in Table 1 for 20 μM stock solution and H_2O to give a total of 180 μL for each respective standard. This schematic will provide sufficient volume for assay in triplicate.

Table 1.

Standard #	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Concentration (μM)	0	0.1	0.2	0.5	1	2	3	4
20 μM Stock (μL)	0	5	9	22	45	90	135	180
H_20 (μL)	180	175	171	158	135	90	45	0

ASSAY PROCEDURE

NOTE: Centrifugation is absolutely necessary due to the presence of a precipitate in solution. Be careful not to disturb this pellet after centrifugation, as it will interfere with accurate readings. If the pellet is disturbed, centrifuge the sample again.

Reagent Blank: Create a reagent blank (B_0) to correct for any contribution to the A_{586} due to the chromophore in the sample. A 325 μL blank is made by combining one volume of the provided Diluent with three volumes of acetonitrile. Use this blank in place of the diluted Reagent R1 as described in step 2 of the assay procedures below.

Sample Blank: A sample blank (water substituting for sample) is included in the standard curve preparation (S_1).

1. Add 140 μL of sample, standard, or blank to a clean, labeled polypropylene microcentrifuge tube.
2. Add 455 μL of diluted Reagent R1 (see Reagent Preparation) to each tube.
3. Mix gently by vortexing.
4. **NOTE: For MDA only:** Add 105 μL of 12N HCl (37% HCl) to each tube.
   For MDA + HAE: Add 105 μL of Reagent R2 to each tube.
5. Mix well and close cap.
6. Incubate at 45°C for 60 minutes.
7. Centrifuge turbid samples to obtain clear supernatant (13,000 x *g* for 15 minutes).
8. Transfer 3 x 150 μL of the clear supernatant to a microplate (see template below).
9. Measure absorbance at 586 nm. If this is not available, a 580 nm or 590 nm filter can be used. The color is stable for at least one hour at room temperature or 2 hours at 4°C if the plate is stored in the dark.

Scheme I

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_1	S_1	S_1	B_0	B_0	B_0	U_8	U_8	U_8	U_{16}	U_{16}	U_{16}
B	S_2	S_2	S_2	U_1	U_1	U_1	U_9	U_9	U_9	U_{17}	U_{17}	U_{17}
C	S_3	S_3	S_3	U_2	U_2	U_2	U_{10}	U_{10}	U_{10}	U_{18}	U_{18}	U_{18}
D	S_4	S_4	S_4	U_3	U_3	U_3	U_{11}	U_{11}	U_{11}	U_{19}	U_{19}	U_{19}
E	S_5	S_5	S_5	U_4	U_4	U_4	U_{12}	U_{12}	U_{12}	U_{20}	U_{20}	U_{20}
F	S_6	S_6	S_6	U_5	U_5	U_5	U_{13}	U_{13}	U_{13}	U_{21}	U_{21}	U_{21}
G	S_7	S_7	S_7	U_6	U_6	U_6	U_{14}	U_{14}	U_{14}	U_{22}	U_{22}	U_{22}
H	S_8	S_8	S_8	U_7	U_7	U_7	U_{15}	U_{15}	U_{15}	U_{23}	U_{23}	U_{23}

CALCULATIONS

In HCl and MSA, the molar extinction coefficient (ε) at 586 nm for MDA is approximately 120,000. The color yield is a linear function of the MDA concentration over the range from 0 μM to 20 μM. 4-HNE cannot be measured in the HCl protocol, as it does not form a chromphore with the reagent in the presence of HCl. For simultaneous determination of both MDA and 4-HNE, the MSA procedure must be used. The extinction coefficient using MSA is determined empirically from the slope of a standard curve in a manner similar to that described in the Example.

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]:

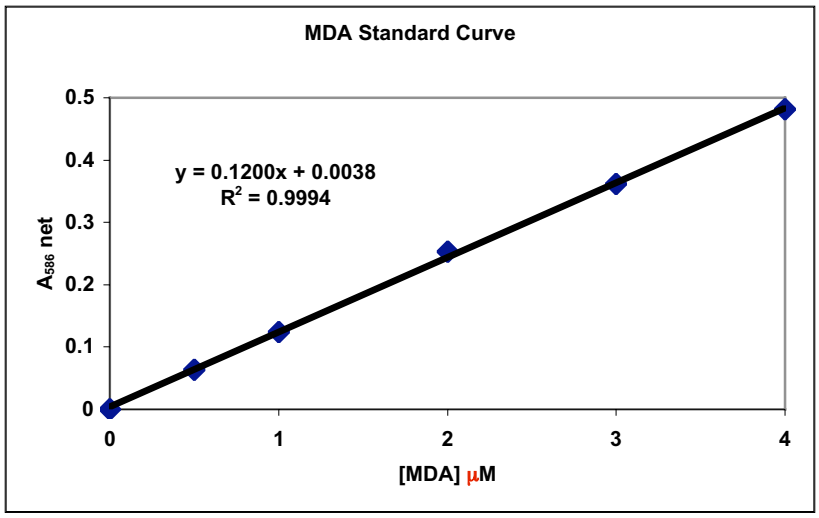
$$[MDA] = a[MDA] + b$$

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.):

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

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: Typical Standard Curve



Example:
To illustrate the appropriate calculations, consider the following experiment:
Three 200 mL aliquots of a biological sample were assayed along with a triplicate set of MDA standards. A sample blank had an absorbance of 0 at 586 nm. The average A_{586} value of the zero concentration standard was subtracted from the average A_{586} values of the other standards and the sample to give corrected absorbencies (A_{586} net). A plot of A_{586} net vs. [MDA] for the standards was constructed (see Figure 1 above).
The sample had an average A_{586} net = 0.2752. Steps in the calculation of sample [MDA] are:

1. Calculate the [MDA] in the reaction mixture:

$$[\text{MDA}] = \frac{0.2752-0.0038}{0.1200} = 2.261\ \mu\text{M}$$

2. Correct for the dilution factor of the sample:
140 mL of sample was used and the final reaction volume was 700 mL.

$$\text{df} = \frac{700}{140} = 5.000$$

[MDA] in biological sample = 2.261 mM x 5.000 = 11.3 mM

PERFORMANCE CHARACTERISTICS

LEAST DETECTABLE CONCENTRATION

Experiments employing enaldehyde standards and blanks suggest that the analytical limit of detection in a purified system is 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. It is recommended that the researcher confirm 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 that the standard error of the measurement (SEM) is less than 5%.

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