Colorimetric Assay for
Lipid Peroxidation

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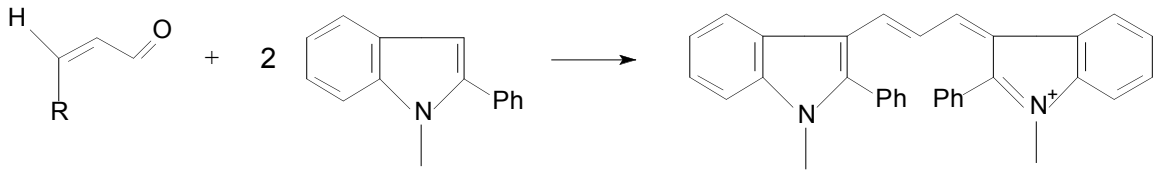
INTRODUCTION

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. The measurement of MDA has been used as an indicator of lipid peroxidation (1). This method is designed to assay MDA.

PRINCIPLES OF PROCEDURE

This assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R1), with MDA at 45°C. One molecule of MDA 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

Component	Description	Volume	Storage
Reagent R1	N-methyl-2-phenylindole in Acetonitrile	3 x 18 mL	4°C
MDA Standard	1,1,3,3-Tetramethoxypropane (TMOP) in Tris-HCl	1 mL	4°C
Diluent	Ferric Iron in Methanol	30 mL	4°C

MATERIALS NEEDED BUT NOT PROVIDED

1. Spectrophotometer with a 586 nm filter
2. Spectrophotometric cuvettes with a 1 cm optical path length
3. Water bath at 45°C
4. Disposable test tubes and stoppers compatible with acetonitrile, methanol, and acid
5. 37% HCl
6. Butylated hydroxytoluene (BHT)
7. Acetonitrile

8. Microcentrifuge
9. Polypropylene microcentrifuge tubes
10. Deionized Water (dH_2O)
11. Adjustable micropipettes (10 – 1000 μL) and tips

Optional:

1. Methanesulfonic Acid

STORAGE

1. The reagents are stable until the indicated kit expiration date if handled and stored properly.
2. When not in use, place the bottles at 4°C.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. In case of accidental exposure of skin, mucous membranes or eyes to R1 reagent, thoroughly wash the exposed area with water.
3. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
2. Do not allow the capped reagent bottles to sit at room temperature for long periods of time.
3. 4-hydroxyalkenals (HAE) plus MDA can be measured by substituting methanesulfonic acid for HCl in **ASSAY PROCEDURE** Step 3 below.

REAGENT PREPARATION

1. **100x BHT Stock Solution:** 0.5 M in Acetonitrile.
2. **Reagent R1:** Dilute Reagent R1 3:1 with Diluent (ex: 18 mL R1 + 6 mL Diluent). Prepare immediately before use.
3. **37% HCl:** 12 N HCl – Do not dilute prior to use in the assay.
4. **Sample Blank:** 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.

SAMPLE PREPARATION

Sample Stability

Unless assayed immediately, samples should be frozen at -70°C to prevent loss of MDA (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.

Oxidation Prevention

We recommend adding BHT at a final concentration of 5 mM in the buffer prior to homogenization of tissue or cells. If no antioxidant is added, new lipid peroxidation can occur during homogenization and biased values will result (2).

Plasma or Serum

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

Cell Culture (8,9)

Cells cultured in serum containing medium should be washed several times to remove serum components prior to homogenization. Since MDA exists as the water-soluble enolate anion at physiological pH, much of the MDA generated from lipid peroxidation in cell culture may be in the culture medium.

1. Remove cells using a rubber policeman. Lysis buffers have a high potential of interfering in this assay. Cells should be washed well in ice-cold 20 mM PBS or Tris buffer, 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. Lyse cells by sonication, homogenization, or freeze-thaw cycles. To prevent sample oxidation during preparation, lysis should be done in the presence of 10 μL 0.5 M BHT per 1 ml of cell homogenate.
3. After homogenization, follow steps 5-7 of the tissue homogenization procedure.

Tissue Homogenates (6,7)

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.

1. If necessary, remove blood *in situ* by perfusion or *in vitro* by rinsing with ice-cold isotonic saline (0.9% NaCl).
2. Weigh tissue. A reasonable amount to start with is 1 g tissue per 10 mL of buffer.
3. Prepare tissue homogenate in ice-cold 20 mM PBS or Tris 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.
4. Add 10 μL 0.5 M BHT Stock Solution per 1 mL of tissue homogenate to prevent sample oxidation. A precipitate is expected and will not affect the outcome of the assay as it is removed by centrifugation.
5. After homogenization, centrifuge at 3,000 x *g* and 4°C for 10 minutes to remove large particles.
6. Remove an aliquot of the sample for protein determination.
7. Freeze the sample immediately at -70°C or keep on ice prior to testing. Test 0.2 mL of the homogenate in the assay.

STANDARD CURVE PREPARATION

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 MDA 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.

Use the zero concentration standard (S_0) as a blank to zero the spectrophotometer used to run the assay.

Table 1: Standard Curve Preparation

Standard	MDA Conc. (μM)	Vol. of dH_2O (μL)	Vol. of 20 μM MDA Stock (μL)
S_0	0	200	-
S_1	2.5	175	25
S_2	5.0	150	50
S_3	10.0	100	100
S_4	15.0	50	150
S_5	20.0	-	200

ASSAY PROCEDURE

1. Add 200 μL of Standards or Samples to a microcentrifuge tube.
2. Add 650 μL of diluted Reagent R1 to each tube and vortex.
3. Add 150 μL 37% HCl (12 N HCl) 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 supernatant to a cuvette and read at 586 nm.
   The color is stable for at least an hour at room temperature, or 2 hours at 4°C when stored in the dark.

CALCULATIONS

1. Using the standard data, calculate the net A_{586} for each standard by subtracting the blank (S_0) value from each of the standard A_{586} values. Plot the net A_{586} against MDA concentration, and perform a linear regression analysis:

$$[\mathbf{A_{586}}] = m[\mathbf{MDA}] + b$$

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

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

Where:
[MDA] = μM concentration of MDA in the sample
A_{586} = Net absorbance at 586 nm of the sample
m = regression coefficient (slope)
df = dilution factor

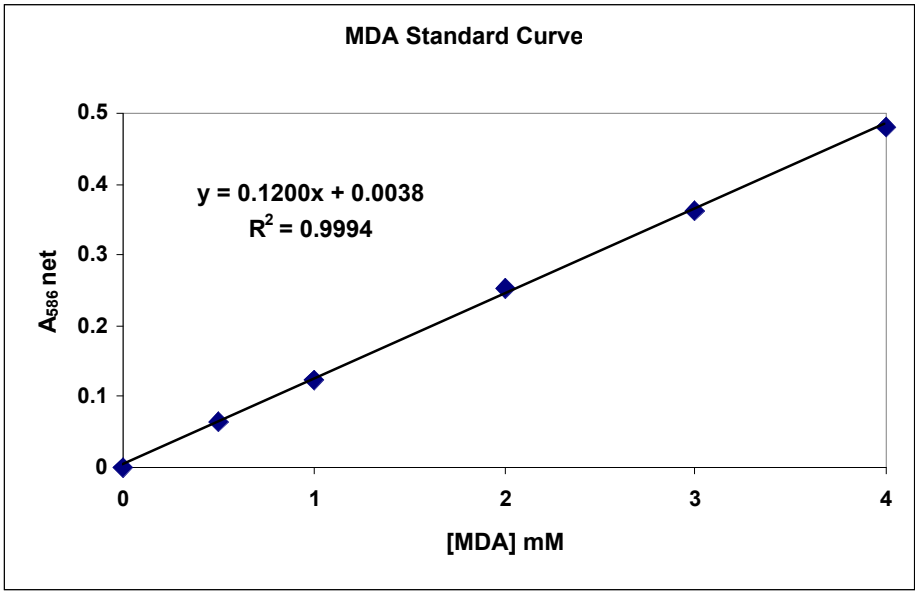


Figure 1: Typical Standard Curve

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%.

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 A_{586}.
3. This assay measures only free MDA in samples. The conditions of the assay do not provide for liberation of MDA bound to proteins via Schiff Base. 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 (2,7). 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 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 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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