Lipid Peroxidation Microplate Assay

Catalog Number: LIP39-K01

96 Wells

For Research Use Only

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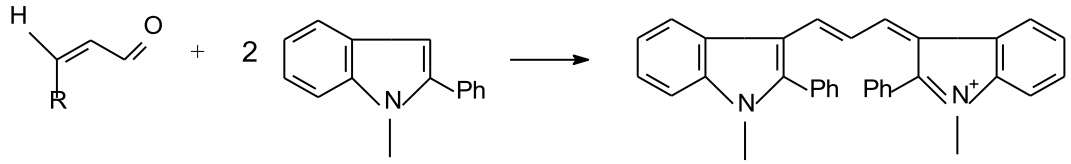
INTENDED USE

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 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.)

For further information about this kit, its application, or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc at www.EagleBio.com or at 866-411-8023.

ASSAY PRINCIPLES

This Lipid Peroxidation 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 **Max = 586 nm**

4-hydroxyalkenal : R= hydroxyalkyl

MATERIALS PROVIDED

Store all other reagents at 2 to 8°C. Use only reagents supplied with this Lipid Peroxidation Assay kit. Do not interchange reagents with different lot numbers. Expiration dates and lot numbers are printed on the labels.

1. **MICROPLATE—**96-well Microplate
2. **MDA STANDARD**—1 vial (200 µL) of 1,1,3,3- Tetramethoxypropane (TMOP) in Tris-HCI
3. **DILUENT**—1 bottle (5.5 mL) of Ferric Iron in Methanol
4. **REAGENT R1**—1 bottle (13.0 mL) of N-methyl-2-phenylindole in Acetronitrile

STORAGE

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

MATERIALS NEEDED BUT NOT PROVIDED

1. Spectrophotometer plate reader with a 586 nm filter (580 nm or 590 nm filters are also acceptable)
2. Water bath at 45°C
3. 37% HCl (optional – used for measuring MDA only)
4. Butylated hydroxytoluene (BHT) (used in sample collection)
5. Acetonitrile
6. Microcentrifuge
7. Polypropylene microcentrifuge tubes
8. Deionized Water (dH_2O)
9. Adjustable micropipettes (10 – 1000 µL) and tips

WARNINGS AND PRECAUTIONS

- Use aseptic technique when opening and dispensing reagents.
- In case of accidental exposure of skin, mucous membranes or eyes to R1 or R2 reagents, thoroughly wash the exposed area with water.
- 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

- Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
- Do not allow the capped reagent bottles to sit at room temperature for long periods of time.

- 4-hydroxyalkenals (HAE) plus MDA can be measured by substituting methanesulfonic acid for HCI in **ASSAY PROCEDURE** step 3 below.
- To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

REAGENT PREPARATION

1. **100x BHT Stock Solution:** 0.5 M in Acetonitrile.
2. **Reagent R1:** Dilute Reagent R1 3:1 with Diluent (i.e. 12 mL R1 + 4 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.
5. **20 µM MDA Standard Stock:** Dilute the 10 mM MDA Standard 1:500 in dH_2O (i.e. 20 µL 10 mM MDA + 9.98 mL dH_2O). Prepare immediately before use.

SAMPLE PREPARATION

Sample Stability

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

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 5×10^7 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.
- Please see the **Reagent Preparation** section for preparing the 20 µM MDA Standard Stock.

Table 1: Standard Curve Preparation

Standard	MDA Conc. (µM)	Vol. of dH_2O (µL)	Vol. of 20 µM MDA Stock (µL)
S0	0	200	-
S1	0.5	195	5
S2	1.0	190	10
S3	2.5	175	25
S4	5.0	150	50
S5	10.0	100	100
S6	15.0	50	150
S7	20.0	-	200

ASSAY PROCEDURE

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 37% HCI (12 N HCI) 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.

Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_0	S_0	BLK	BLK	BLK	U_8	U_8	U_8	U_{16}	U_{16}	U_{16}
B	S_1	S_1	S_1	U_1	U_1	U_1	U_9	U_9	U_9	U_{17}	U_{17}	U_{17}
C	S_2	S_2	S_2	U_2	U_2	U_2	U_{10}	U_{10}	U_{10}	U_{18}	U_{18}	U_{18}
D	S_3	S_3	S_3	U_3	U_3	U_3	U_{11}	U_{11}	U_{11}	U_{19}	U_{19}	U_{19}
E	S_4	S_4	S_4	U_4	U_4	U_4	U_{12}	U_{12}	U_{12}	U_{20}	U_{20}	U_{20}
F	S_5	S_5	S_5	U_5	U_5	U_5	U_{13}	U_{13}	U_{13}	U_{21}	U_{21}	U_{21}
G	S_6	S_6	S_6	U_6	U_6	U_6	U_{14}	U_{14}	U_{14}	U_{22}	U_{22}	U_{22}
H	S_7	S_7	S_7	U_7	U_7	U_7	U_{15}	U_{15}	U_{15}	U_{23}	U_{23}	U_{23}

CALCULATIONS

1. Using the standard data, calculate the net A_{586} for each standard by subtracting the blank (S0) value from each of the standard A_{586} values. Plot the net A_{586} against MDA concentration, and perform a linear regression analysis:

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

2. Calculate the concentration of analyte in each unknown from 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:

$$[MDA] = \frac{A^{586} - b}{m} \cdot df$$

Where: [MDA] = µM concentration of MDA in the sample

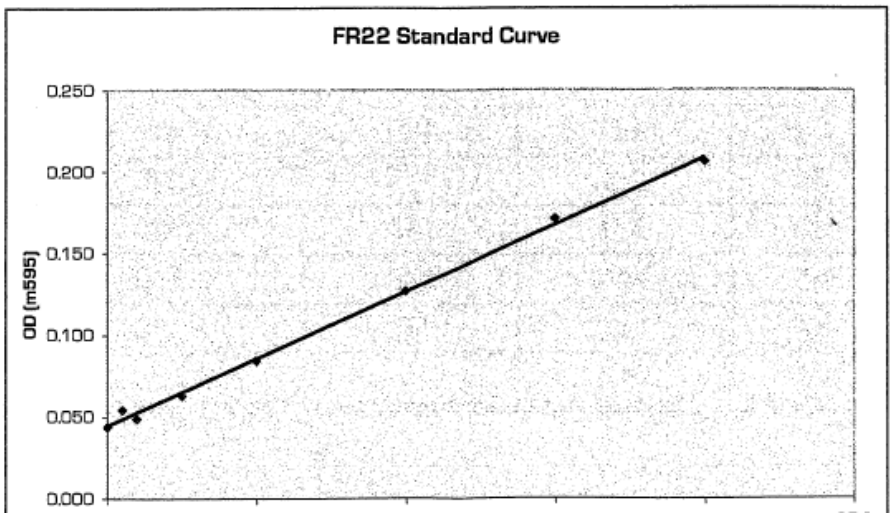
A_{586} = Net absorbance at 586 nm of the sample

m=regression coefficient (slope)

b = intercept

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

- 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.
- 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}.
- 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. 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).
- 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.
- 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.
- 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.

- If no antioxidant is added to the samples during homogenization and subsequent assay, a high bias due to new sample oxidation may result.

PRECAUTIONS

- This kit is for research use only
- Compare contents and packing list, if there is breakage or shortage, notify Eagle Biosciences immediately
- Do not pipette reagents by mouth
- Do not smoke, eat or drink while performing assay
- Wear disposable gloves and proper lab protection and attire
- Treat all samples as potentially infectious.
- Do not mix reagents from other lots
- Avoid contact with TMB and Stop solutions. If contact occurs, rinse thoroughly with water
- Eagle Biosciences is not responsible for outcomes as results of tampering with the reagents or using them unconventionally

REFERENCES

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Product Developed and Manufactured in the USA.