TBARS Assay Kit

Catalog Number KA1381

96 assays

Version: 11

Intended for research use only
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Introduction

Background

Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. 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.\(^1\)\(^2\) Lipid peroxides, derived from polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as MDA. In human platelets, thromboxane synthase also catalyzes the conversion of PGH\(_2\) to thromboxane A\(_2\), 12(S)-HHT\(\alpha\)E, and MDA in a ratio of 1:1:1.\(^3\)

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established method for screening and monitoring lipid peroxidation.\(^1\)\(^2\) Modifications of the TBARS assay by many researchers have been used to evaluate several types of samples including human and animal tissues and fluids, drugs, and foods.\(^4\)\(^6\) Even though there remains a controversy cited in literature regarding the specificity of TBARS toward compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation.\(^2\) If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized, and the test becomes quite specific for lipid peroxidation.\(^2\) Lipids with greater unsaturation will yield higher TBARS values. It is recommended that if high TBARS values are obtained, a more specific assay such as HPLC should be performed.

Principle of the Assay

TBARS Assay Kit provides a simple, reproducible, and standardized tool for assaying lipid peroxidation in plasma, serum, urine, tissue homogenates, and cell lysates. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions is measured colorimetrically at 530-540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Although this reaction has a much higher sensitivity when measured fluorometrically, protocols for both methods are provided.
General Information

Materials Supplied

List of component

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiobarbituric Acid</td>
<td>1 vial</td>
</tr>
<tr>
<td>TBA Acetic Acid</td>
<td>2 vials</td>
</tr>
<tr>
<td>TBA Sodium Hydroxide (10X)</td>
<td>1 vial</td>
</tr>
<tr>
<td>TBA Malondialdehyde Standard</td>
<td>1 vial</td>
</tr>
<tr>
<td>TBA SDS Solution</td>
<td>1 vial</td>
</tr>
<tr>
<td>96-Well Solid Plate (Colorimetric Assay)</td>
<td>1 plate</td>
</tr>
<tr>
<td>96-Well Solid Plate (black)</td>
<td>1 plate</td>
</tr>
<tr>
<td>96-Well Cover Sheet</td>
<td>2 covers</td>
</tr>
</tbody>
</table>

Storage Instruction

This kit will perform as specified if stored at 4°C and used before the expiration date indicated on the outside of the box.

Materials Required but Not Supplied

✓ A plate reader capable of measuring absorbance between 530-540 nm or a fluorometer with the capacity to measure fluorescence using an excitation wavelength of 530 nm and an emission wavelength of 550 nm
✓ Adjustable pipettes and a repeating pipettor
✓ A source of pure water. Glass distilled water or HPLC-grade water is acceptable
✓ Container sufficient to boil samples and standards
✓ 5 mL polypropylene screw-cap centrifuge tubes
✓ Centrifuge capable of spinning 5 mL centrifuge tubes at 1,600 x g at 4°C
**Precautions for Use**

- **Precaution**
  ✓ This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.
  ✓ Please read these instructions carefully before beginning this assay.
  ✓ It is recommended to take appropriate precautions when using the kit reagents (i.e., lab coat, gloves, eye goggles, etc.), as some of them can be harmful.
  ✓ The sodium hydroxide and acid solutions are corrosive and harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes.
  ✓ Care should be exercised when removing samples from boiling water.

- **Pipetting hint**
  ✓ It is recommended that an adjustable pipette be used to deliver reagents to the wells.
  ✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
  ✓ Do not expose the pipette tip to the reagent(s) already in the well.

- **Plate setup**
  There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in duplicate is shown below in Plate Layout.
Assay Protocol

Reagent Preparation

- Standard
- Colorimetric Standard Preparation
  Dilute 250 μL of the MDA standard with 750 μL of water to obtain a stock solution of 125 μM. Take eight clean glass test tubes and label them A-H. Add the amount of 125 μM MDA stock solution and water to each tube as described in Table 1. (MDA colorimetric standards)

<table>
<thead>
<tr>
<th>Tube</th>
<th>MDA (μL)</th>
<th>Water (μL)</th>
<th>MDA Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1,000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>995</td>
<td>0.625</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>990</td>
<td>1.25</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>980</td>
<td>2.5</td>
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<tr>
<td>E</td>
<td>40</td>
<td>960</td>
<td>5</td>
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<td>F</td>
<td>80</td>
<td>920</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>200</td>
<td>800</td>
<td>25</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>600</td>
<td>50</td>
</tr>
</tbody>
</table>

- Fluorometric Standard Preparation
  Dilute 25 μL of the MDA standard with 975 μL of water to obtain a stock solution of 12.5 μM. Take eight clean glass test tubes and label them A-H. Add the amount of 12.5 μM MDA stock solution and water to each tube as described in Table 2. (MDA fluorometric standards)

<table>
<thead>
<tr>
<th>Tube</th>
<th>MDA (μL)</th>
<th>Water (μL)</th>
<th>MDA Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1,000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>995</td>
<td>0.0625</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>990</td>
<td>0.125</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>980</td>
<td>0.25</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>960</td>
<td>0.5</td>
</tr>
<tr>
<td>F</td>
<td>80</td>
<td>920</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>200</td>
<td>800</td>
<td>2.5</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>600</td>
<td>5</td>
</tr>
</tbody>
</table>

- Thiobarbituric Acid
  The vial contains 2 g of thiobarbituric acid (TBA). It is ready to use to prepare the Color Reagent.

- TBA Acetic Acid
  Each vial contains 20 mL of concentrated acetic acid. Slowly add both vials (40 mL) of TBA Acetic Acid to 160 mL of HPLC-grade water. This diluted acetic acid solution is used in preparing the color reagent. The
diluted acetic acid solution is stable for at least three months at room temperature.

- **TBA Sodium Hydroxide (10X)**
The vial contains a solution of sodium hydroxide (NaOH). Dilute 20 mL of TBA NaOH with 180 mL of HPLC-grade water. This diluted NaOH solution is used in preparing the color reagent. The diluted NaOH solution is stable for at least three months at room temperature. Store the diluted NaOH solution in a plastic container suitable for corrosive materials.

- **TBA Malondialdehyde Standard**
The vial contains 500 µM Malondialdehyde (MDA) in water. It is ready to use to prepare the standard curve.

- **TBA SDS Solution**
The vial contains a solution of sodium dodecyl sulfate (SDS). The solution is ready to use as supplied.

- **To prepare the Color Reagent**
The following amount of color reagent is sufficient to evaluate 24 samples. Adjust the volumes accordingly if more or less samples are going to be assayed. Weigh 530 mg of TBA and add to ≥150 mL beaker containing 50 mL of diluted TBA Acetic Acid solution. Add 50 mL of diluted TBA Sodium Hydroxide and mix until the TBA is completely dissolved. The solution is stable for 24 hours.

**Sample preparation**

- **Plasma**
Typically, normal human plasma has a lipid peroxide level (expressed in terms of MDA) of 1.86-3.94 µM.¹,²
  - Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
  - Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. If not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
  - Plasma does not need to be diluted before assaying.

- **Serum**
Typically, normal human serum has a lipid peroxide level (expressed in terms of MDA) of 1.86-3.94 µM.¹
  - Collect blood without using an anticoagulant.
  - Allow blood to clot for 30 minutes at 25°C.
  - Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
  - Serum does not need to be diluted before assaying.
- **Urine**

  Typically, normal human urine has a lipid peroxide level (expressed in terms of MDA) of 0.8-2 μmol/g creatinine.⁹,¹⁰

  ✓ Urine does not require any special treatments. If not assaying the same day, freeze at -80°C.

- **Tissue Homogenates**

  ✓ Weigh out approximately 25 mg of tissue into a 1.5 mL centrifuge tube.
  ✓ Add 250 μL of RIPA buffer Concentrate with protease inhibitors of choice.
  ✓ Sonicate for 15 seconds at 40V over ice.
  ✓ Centrifuge the tube at 1,600 x g for 10 minutes at 4°C. Use the supernatant for analysis. Store supernatant on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for one month.
  ✓ Tissue homogenates do not need to be diluted before assaying.

- **Cell Lysates**

  ✓ Collect 2 x 10⁷ cells in 1 mL of cell culture medium or buffer of choice, such as PBS.
  ✓ Sonicate 3X for five second intervals at 40V setting over ice.
  ✓ Use the whole homogenate in the assay, being sure to use the culture medium as a sample blank.
  ✓ Cell lysates do not need to be diluted before assaying.

**Assay Procedure**

- **General Information**

  ✓ All reagents except samples must be equilibrated to room temperature before beginning the assay. The SDS Solution will take at least one hour to equilibrate to room temperature if stored at 2-8°C. Briefly heating the SDS Solution at 37°C will re-dissolve the precipitated SDS. The SDS Solution can then be stored at room temperature.
  ✓ The final volume of the assay is 150 μL in all wells.
  ✓ The assay is performed at room temperature.
  ✓ It is not necessary to use all the wells on the plate at one time.
  ✓ It is recommended that the samples and standards be assayed at least in duplicate.
  ✓ It is recommended that the samples and standards be kept at 4°C after preparation to increase sensitivity and reproducibility.
  ✓ Monitor the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.

1. Label vial caps with standard number or sample identification number.

2. Add 100 μL of sample or standard to appropriately labeled 5 mL vial.
3. Add 100 μL of SDS solution to vial and swirl to mix.
4. Add 4 mL of the Color Reagent forcefully down side of each vial.
5. Cap vials and place vials in foam or some other holder to keep the tubes upright during boiling.
6. Add vials to vigorously boiling water. Boil vials for one hour.
7. After one hour, immediately remove the vials and place in ice bath to stop reaction. Incubate on ice for 10 minutes.
8. After 10 minutes, centrifuge the vials for 10 minutes at 1,600 x g at 4°C. Vials may appear clear or cloudy. Cloudiness will clear upon warming to room temperature.
9. Vials are stable at room temperature for 30 minutes.
10. Load 150 μL (in duplicate) from each vial to either the clear plate (colorimetric version) or to the black plate (fluorometric version).
11. Read the absorbance at 530-540 nm or read fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm.
Data Analysis

Calculation of Results

- Colorimetric Calculations
  - Calculate the average absorbance of each standard and sample.
  - Subtract the absorbance value of the standard A (0 µM) from itself and all other values (both standards and samples). This is the corrected absorbance.
  - Plot the corrected absorbance values (from step 2 above) of each standard as a function of MDA concentration.
  - Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown below.

\[
MDA (\mu M) = \left( \frac{\text{Corrected absorbance} - (y - \text{intercept})}{\text{Slope}} \right)
\]

Figure 1. MDA colorimetric standard curve

- Fluorometric Calculations
  - Calculate the average fluorescence of each standard and sample.
  - Subtract the fluorescence value of the standard A (0 µM) from itself and all other values (both standards and samples). This is the corrected fluorescence.
  - Plot the corrected fluorescence values (from step 2 above) of each standard as a function of MDA concentration.
Calculate the values of MDA for each sample from the standard curve. An example of the MDA standard curve is shown below.

\[
\text{MDA (µM)} = \frac{(\text{Corrected fluorescence}) - (y \text{- intercept})}{\text{Slope}}
\]

![MDA fluorometric standard curve](image)

**Figure 2.** MDA fluorometric standard curve

**Performance Characteristics**

- **Precision**
  When a series of ten human plasma and sixteen human urine samples were assayed on the same day, the intra-assay coefficient of variation was 5.5% and 7.6%, respectively. When a series of eight human plasma and sixteen human urine samples were assayed on seven different days under the same experimental conditions, the inter-assay coefficient of variation was 5.9% and 5.1%, respectively.

- **Assay Range:**
  Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-50 µM (Colorimetric) or 0-5 µM (Fluorometric) (µM = µmole/liter = nmol/mL) MDA equivalents.
**Interferences**

The following reagents were tested for interference in the assay.

<table>
<thead>
<tr>
<th></th>
<th>Will Interfere (Yes or No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffers:</strong></td>
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</tr>
<tr>
<td>Borate (50 mM)</td>
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<tr>
<td>HEPES (100 mM)</td>
<td>No</td>
</tr>
<tr>
<td>Phosphate (100 mM)</td>
<td>No</td>
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<tr>
<td>Tris (25 mM)</td>
<td>No</td>
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<tr>
<td><strong>Detergents:</strong></td>
<td></td>
</tr>
<tr>
<td>CHAPS (≤1%)</td>
<td>No</td>
</tr>
<tr>
<td>Triton X-100 (≤1%)</td>
<td>No</td>
</tr>
<tr>
<td>Tween 20 (≤1%)</td>
<td>No</td>
</tr>
<tr>
<td><strong>Protease Inhibitors/Chelators:</strong></td>
<td></td>
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<tr>
<td>Antipain (≤0.1 mg/mL)</td>
<td>No</td>
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<tr>
<td>Chymostatin (≤10 µg/mL)</td>
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<td>Leupeptin (≤10 µg/mL)</td>
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<td>PMSF (≤200 µM)</td>
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<td>Trypsin (≤10 µg/mL)</td>
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<td>EDTA (≤1 mM)</td>
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<td>EGTA (≤1 mM)</td>
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<td><strong>Others:</strong></td>
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<td>Sucrose (250 mM)</td>
<td>Yes</td>
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<td>Glycerol (≤10%)</td>
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Resources

Trouble shooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Causes</th>
<th>Recommended Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erratic values; dispersion of duplicates/triplicates</td>
<td>A. Poor pipetting/technique</td>
<td>A. Be careful not to splash the contents of the wells</td>
</tr>
<tr>
<td></td>
<td>B. Bubble in the well(s)</td>
<td>B. Carefully tap the side of the plate with your finger to remove bubbles</td>
</tr>
<tr>
<td>No MDA was detected in the sample</td>
<td>A. MDA concentration was too low</td>
<td>A. Process more tissue (50-100 mg)</td>
</tr>
<tr>
<td></td>
<td>B. The sample was too dilute</td>
<td>B. Harvest more cells (2 x 10^8) and re-assay</td>
</tr>
<tr>
<td>The fluorometer exhibited ‘MAX’ values for the wells</td>
<td>The GAIN setting is too high</td>
<td>Reduce the GAIN and re-read</td>
</tr>
</tbody>
</table>

References


<table>
<thead>
<tr>
<th></th>
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<th>2</th>
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<tbody>
<tr>
<td>A</td>
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