ab118970
Lipid Peroxidation (MDA) Assay kit (Colorimetric/Fluorometric)

Instructions for use:

For the rapid, sensitive and accurate measurement of MDA (end product of lipid peroxidation) in a variety of samples.

This product is for research use only and is not intended for diagnostic use.

Version 9 Last Updated 10 May 2016
1. BACKGROUND

Lipid Peroxidation Assay Kit (Colorimetric/Fluorometric) (ab118970) is a convenient tool for sensitive detection of malondialdehyde (MDA) present in a variety of samples. The free MDA present in the sample reacts with Thiobarbituric Acid (TBA) to generate a MDA-TBA adduct, which can be easily quantified colorimetrically (OD 532 nm) or fluorometrically (Ex/Em = 532/553 nm). This assay can detect as low as 0.1 nmol/well MDA in the fluorometric assay (1 nmol/well MDA in colorimetric assay).

Lipid peroxidation refers to the oxidative degradation of lipids. In this process free radicals take electrons from the lipids (generally in cell membranes), resulting in cell damage. Quantification of lipid peroxidation is essential to assess oxidative stress in pathophysiological processes. The end products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), as natural bi-products. Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage.
2. ASSAY SUMMARY

Sample preparation

Standard curve preparation

Add TBA solution

Measure optical density (OD532 nm) or fluorescence (Ex/Em= 532/553 nm)
3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.
5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA Lysis Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Phosphotungstic Acid Solution</td>
<td>12.5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>BHT (100X)</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>TBA Solution</td>
<td>4 vials</td>
<td>-20°C</td>
<td>4°C</td>
</tr>
<tr>
<td>MDA Standard (4.17 M)</td>
<td>100 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
GENERAL INFORMATION

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 532 nm or fluorescence at Ex/Em = 532/553 nm.
- MilliQ water or other type of double distilled water (ddH₂O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom (colorimetric assay) / 96 well plate with clear flat bottom, preferably black (fluorometric assay)
- Dounce homogenizer (if using tissue)
- Glacial Acetic Acid
- 42 mM H₂SO₄ – for plasma sample preparation
- (Optional) Sonicator – to help solubilize TBA solution
- (Optional) N-Butanol – to increase assay sensitivity
- (Optional) 5M NaCl – to increase assay sensitivity
- (Optional) 2N perchloric acid – for alternative cell/tissue preparation protocol
8. TECHNICAL HINTS

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.

- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.

- Ensure plates are properly sealed or covered during incubation steps.

- Make sure you have the right type of plate for your detection method of choice.

- Make sure all necessary equipment is switched on and set at the appropriate temperature.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening

9.1. MDA Lysis Buffer:
Ready to use as supplied. Equilibrate to room temperature before use. Aliquot buffer so that you have enough to perform the desired number of assays. Store at -20°C.

9.2. Phosphotungstic Acid Solution:
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3. BHT (100X):
Ready to use as supplied. Equilibrate to room temperature before use. Aliquot solution so that you have enough to perform the desired number of assays. Store at -20°C.

9.4. TBA Solution:
Reconstitute one vial of TBA in 7.5 mL Glacial Acetic Acid. Transfer slurry to another tube and then adjust the final volume to 25 mL with ddH₂O. Mix well to dissolve. Sonication in a RT water bath can be used if required. Store at 4°C. Reconstituted TBA solution is stable for 1 week.

9.5. MDA Standard (4.17M):
Ready to use as supplied. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C.
10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Discard the working standard dilutions after use as they do not store well.

10.1. For colorimetric assay:

10.1.1. Prepare a 0.1 M MDA standard by diluting 10 µL 4.17M MDA Standard in 407 µL of ddH₂O.

10.1.2. Prepare a 2 mM MDA standard by diluting 10 µL 0.1 M MDA Standard in 490 µL of ddH₂O.

10.1.3. Using 2 mM MDA standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of MDA Standard (µL)</th>
<th>Volume ddH₂O (µL)</th>
<th>Final Volume standard in well (µL)</th>
<th>End Conc MDA in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>600</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>594</td>
<td>200</td>
<td>4 (20 µM)</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>588</td>
<td>200</td>
<td>8 (40 µM)</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>582</td>
<td>200</td>
<td>12 (60 µM)</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>576</td>
<td>200</td>
<td>16 (80 µM)</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>570</td>
<td>200</td>
<td>20 (100 µM)</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate reading (2 x 200 µL).
10.2. **For fluorometric assay:**

10.2.1. Prepare a 0.1 M MDA standard by diluting 10 µL 4.17M MDA Standard in 407 µL of ddH$_2$O.

10.2.2. Prepare a 2 mM MDA standard by diluting 10 µL 0.1 M MDA Standard in 490 µL of ddH$_2$O.

10.2.3. Prepare a 0.2 mM standard by diluting 10 µL 2 mM Standard in 90 µL of ddH$_2$O.

10.2.4. Using 0.2 mM MDA standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

<table>
<thead>
<tr>
<th>Standard #</th>
<th>Volume of MDA Standard (µL)</th>
<th>Volume ddH$_2$O (µL)</th>
<th>Final volume standard in well (µL)</th>
<th>End Conc MDA in well (nmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>600</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>594</td>
<td>200</td>
<td>0.4 (2 µM)</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>588</td>
<td>200</td>
<td>0.8 (4 µM)</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>582</td>
<td>200</td>
<td>1.2 (6 µM)</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>576</td>
<td>200</td>
<td>1.6 (8 µM)</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>570</td>
<td>200</td>
<td>2.0 (10 µM)</td>
</tr>
</tbody>
</table>

Each dilution has enough amount of standard to set up duplicate reading (2 x 200 µL).
11. SAMPLE PREPARATION

General Sample Information

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1. Cells (adherent or suspension) samples:

   11.1.1. Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).
   11.1.2. Wash cells with cold PBS.
   11.1.3. Prepare Lysis Solution: 300 µL of the MDA Lysis Buffer with 3 µL BHT (100X).
   11.1.4. Homogenize cells in 303 µL Lysis Solution (Buffer + BHT) using a Dounce homogenizer (10-50 passes) on ice, until efficient lysis is confirmed, by viewing cells under a microscope.
   11.1.5. Centrifuge at 13,000 x g for 10 minutes to remove insoluble material. Collect supernatant.

For samples containing high amount of proteins, we recommend following the alternative protocol described in section 11.3.

11.2. Tissue samples:

   11.2.1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
   11.2.2. Wash tissue in cold PBS.
11.2.3. Prepare Lysis Solution: 300 µL of the MDA Lysis Buffer with 3 µL BHT (100X).

11.2.4. Homogenize tissue in 303 µL Lysis Solution (Buffer + BHT) with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5. Centrifuge at 13,000 x g for 10 minutes to remove insoluble material. Collect supernatant.

For samples containing high amount of proteins, we recommend following the alternative protocol described in section 11.3.

11.3. Alternative protocol for cells and tissue:

11.3.1. Precipitate protein by homogenizing 10 mg sample in 150 µL ddH₂O + 3 µL BHT.

11.3.2. Add 1 volume of 2N perchloric acid.

11.3.3. Vortex and then centrifuge to remove precipitated protein.

11.3.4. Place 200 µL of the supernatant from each sample into a microcentrifuge tube.

11.4. Plasma:

11.4.1. Gently mix 20 µL plasma with 500 µL of 42 mM H₂SO₄ in a microcentrifuge tube.

11.4.2. Add 125 µL of Phosphotungstic Acid Solution and mix by vortexing.

11.4.3. Incubate at room temperature for 5 minutes.

11.4.4. Centrifuge at 13,000 x g for 3 minutes.

11.4.5. Collect the pellet and resuspend on ice with 100 µL ddH₂O (with 2 µL BHT (100X)).

11.4.6. Adjust the final volume to 200 µL with ddH₂O.

**NOTE:** *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*
12. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to correct temperature prior to use.
- We recommended to assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- Follow procedure for enhanced sensitivity when working with plasma and other samples where low MDA is expected.

12.1. Generation of MDA-TBA adduct:

12.1.1. Add 600 µL of TBA reagent into each vial or well containing 200 µL standard and 200 µL sample.

12.1.2. Incubate at 95°C for 60 minutes. Cool to room temperature in an ice bath for 10 minutes.

**NOTE:** Occasionally samples will exhibit a turbidity which can be eliminated by filtering them through a 0.2 µm filter. TBA can react with other compounds in the samples giving other colored compounds. These should not interfere with quantitation of the TBA-MDA adduct.

12.1.3. Take 200 µL supernatant (containing MDA-TBA adduct). and place into a 96-well microplate for analysis.

   Standard (from TBA/Standard mix) = 200 µL
   Sample (from TBA/Sample mix) = 200 µL

12.1.4. Proceed to step 12.3.

12.2. OPTIONAL step for enhanced sensitivity:

**NOTE:** Use this step for plasma and other samples where MDA-TBA adduct concentration is low. After performing n-butanol precipitation step, transfer sample to a black plate and perform fluorometric measurement.
12.2.1. Add 300 µL n-butanol to the 800 µL extract from Step 12.1.2 to precipitate MDA-TBA adduct. If there is no separation, add 100 µL of 5 M NaCl to the mixture and vortex vigorously.

12.2.2. Centrifuge 3 minutes at 16,000 x g at RT. Transfer top layer (containing n-butanol) to a new tube.

12.2.3. Evaporate n-butanol by freeze-dry or by incubating sample in a dry-oven at 55°C.

12.2.4. Dissolve the MDA-TBA adduct in 200 µL ddH₂O and then place into the 96-well plate microplate for analysis.

Standard (from TBA/Standard mix) = 200 µL
Sample (from TBA/Sample mix) = 200 µL

12.3. Measurement:

12.3.1. Measure output immediately on a microplate reader at OD 532 nm for colorimetric assay and at Ex/Em = 532/553 nm for fluorometric assay.

**NOTE:** for fluorometric assay, we recommend to set the instrument sensitivity to high with a slit width of 5 nm.
13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1. Average the duplicate reading for each standard and sample.

13.2. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3. Plot the corrected absorbance values for each standard as a function of the final concentration of MDA.

13.4. Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5. Concentration of MDA in the test samples is calculated as:

\[
MDA\,\text{Concentration} = \left(\frac{A}{mg\ or\ mL}\right) \times 4 \times D
\]

Where:

A = Amount MDA in sample calculated from the standard curve (nmol).

mg = Original tissue amount used (mg).

mL = Original plasma volume used (mL).

4 = correction for using 200 µL of the 800 µL Reaction Mix.

D = Sample dilution factor (if any has been done BEFORE original amount or volume).

For tissue samples, concentration can also be expressed in nmol/mg or nmol/µg of protein if a protein quantification detection has been performed.
14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Figure 1: Typical MDA standard calibration curve using colorimetric reading.

Figure 2: Typical MDA standard calibration curve using fluorometric reading.
Figure 3: Measurement of MDA in human plasma (20 µl) and rat liver lysate (10 mg).
15. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Solubilize TBA, thaw all other components (aliquot if necessary); get equipment ready.
- Prepare MDA standard dilution for your desired detection method: colorimetric [4 – 20 nmol/well] or fluorometric [0.4 – 2 nmol/well].
- Prepare samples in optimal dilutions so that they fit standard curve readings.
- Add 600 µL of TBA solution to 200 µL standard and 200 µL test samples.
- Incubate TBA-standard/TBA-sample mixture at 95°C for 60 minutes. Cool to room temperature in an ice bath for 10 minutes.
- Pipette 200 µL from each 800 µL TBA-standard and TBA-sample reaction mixture into a 96 well microplate.
- OPTIONAL STEP for HIGHER SENSITIVITY: MDA-TBA adduct extraction with 300 µL n-butanol. Dissolve MDA-TBA adduct in 200 µL ddH₂O for analysis.
- Measure plate immediately at OD 532 nm for colorimetric assay or Ex/Em = 532/553 nm for fluorometric assay.
### 16. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Colorimetric: Clear plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorometric: black wells/clear bottom plate</td>
</tr>
</tbody>
</table>

| Sample with erratic readings               | Samples not deproteinized (if indicated on protocol) | Use PCA precipitation protocol for deproteinization         |
|                                            | Cells/tissue samples not homogenized completely  | Use Dounce homogenizer, increase number of strokes          |
|                                            | Samples used after multiple free/thaw cycles    | Aliquot and freeze samples if needed to use multiple times |
|                                            | Use of old or inappropriately stored samples   | Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use |
|                                            | Presence of interfering substance in the sample | Check protocol for interfering substances; deproteinize samples |

| Lower/ Higher readings in samples and Standards | Improperly thawed components | Thaw all components completely and mix gently before use |
|                                                | Allowing reagents to sit for extended times on ice | Always thaw and prepare fresh reaction mix before use |
|                                                | Incorrect incubation times or temperatures   | Verify correct incubation times and temperatures in protocol |

| Standard readings do not follow a linear pattern | Pipetting errors in standard or reaction mix | Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible |
|                                                  | Air bubbles formed in well                  | Pipette gently against the wall of the tubes               |
## RESOURCES

<table>
<thead>
<tr>
<th>Unanticipated results</th>
<th>Standard stock is at incorrect concentration</th>
<th>Always refer to dilutions described in the protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
<td></td>
</tr>
<tr>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
<td></td>
</tr>
<tr>
<td>Sample readings above/ below the linear range</td>
<td>Concentrate/ Dilute sample so it is within the linear range</td>
<td></td>
</tr>
</tbody>
</table>
17. FAQs

Could you explain the steps to fully dissolve the TBA solution if there is precipitate after bringing up the volume to 25 mL with water?

Sonication in a RT water bath can be used if needed. You can also dissolve the TBA in 15 mL of 50% glacial acetic acid in water. Then make up the volume to 25 mL with water. Mix well. This should help in dissolving the TBA. If there is still little precipitate, use 600 µL as directed and any particle in suspension will dissolve at 95°C when incubated for 60 minutes.

Can urine samples be used with this kit?

Urine samples can be used directly with this kit. The samples should be assayed immediately after collection for best results. If the assay is to be performed later, the samples need to be stored at -70°C.

What is the lower detection limit for this assay?

The assay can measure down to 1 nmol per well colorimetrically and 0.1 nmol per well fluorometrically.

When resuspending the pellet from plasma samples with the water/BHT solution, it does not dissolve. Is there a way to better resuspend it?

If you have difficulty resuspending the pellet in the 100 µL ddH₂O (with 2 µL BHT), after the final volume adjust to 200 µL with ddH₂O, the pellet should be solubilized. You can use mild water bath sonication to dissolve the pellet once you have pipetted up and down to break up the initial pellet.

If there is a precipitate in the TBA solution after the 95°C incubation after the assay reaction, is there loss of MDA there, or will it all be in the MDA-TBA adduct in the solution?

If there is turbidity or precipitate formation after heating with TBA, filter the sample with a 0.2 µm filter as mentioned on the protocol.
Why do we use the pellet from plasma samples but the supernatant from tissue samples?

For tissues, PCA precipitates all proteins, and the lipids are in the supernatant. For plasma samples, phosphotungtic acid precipitates lipids and hence the pellet is used.

Is there any major difference between the two methods for preparing the sample (homogenization in MDA lysis buffer vs protein precipitation with perchloric acid)?

The lysis buffer will help disintegrate tissue and lyse cells and since it has SDS, it will denature proteins. Then, once homogenized, the lysate can be centrifuged (debris, nuclei and proteins precipitate). If the tissue is difficult to homogenize in water, we would suggest using this method. Adding PCA will precipitate proteins in the alternative method. Either could work equally well. Effective homogenization is key.

Does mild hemolysis of the plasma affect this assay?

Acid precipitation will remove all proteins including hemoglobin. Moreover, after the addition of PTA solution and centrifugation, the pellet is collected and dissolved in water before being used for the assay. The amount of sample added per well should retain none or insignificant amounts of hemoglobin and this should not affect the readings. Also, the fluorometric method is not affected by hemolysis.

Does this kit detect free MDA, bound-MDA or total MDA?

Effectively, it detects MDA which is free to form an adduct with TBA. If MDA in the sample is bound to collagen or other proteins, this will not be detected unless released. The acid treatment precipitates all proteins, so we expect the MDA in the sample to be free and hence total MDA can be detected.
18. NOTES
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