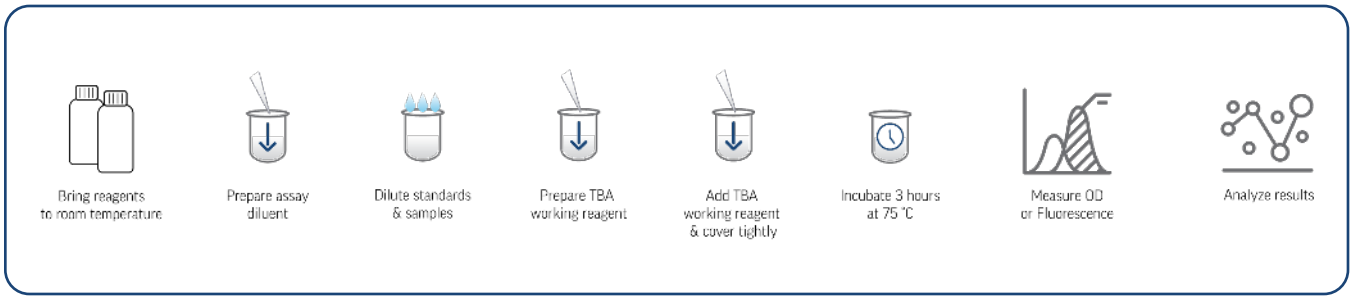
TBARS MALONDIALDEHYDE OXIDATIVE STRESS ASSAY



DESCRIPTION

The TBARS Malondialdehyde Oxidative Stress Assay Kit measures thiobarbituric acid reactive substances in biological samples.

Technical Background

Reactive oxygen species (ROS) such as hydroxyl radicals (·OH), superoxide anions (O^{-2}), or hydrogen peroxide (H_2O_2) are primarily a consequence of oxidative metabolism in mitochondria. Under normal cellular conditions, the radicals are reduced by molecules such as superoxide dismutase (SOD), catalase (CAT), antioxidants like vitamin E, etc. However, conditions that affect the balance of ROS to reducing molecules can damage cellular DNA, RNA, proteins, and lipids.

Lipid peroxidation occurs when ROS remove a hydrogen from the fatty acid moiety of the lipid, which in turn, alters the biological membrane structure, permeability, and function. Upon metabolic breakdown of the altered lipids, "secondary messengers" such as malondialdehyde (MDA), 4-hydroxynonenal, and others form. These serve as biomarkers of dysregulated cellular processes, which have been linked to conditions such as premature birth disorders, diabetes, adult respiratory distress syndromes, shock, Parkinson's disease, Alzheimer's disease, chronic inflammatory conditions, aging, and ischemia-reperfusion mediated injury to kidney, heart, brain, or intestine (1). Lipid peroxidation and release of MDA can also be a marker of animal-based food spoilage (2).

Assay Principle

The TBARS Assay is a quantitative tool for determining TBA reacting substances in biological specimens. Dilutions of standard (MDA) and samples are reacted with TBA in 50% acetic acid at 75 °C for 3 hours. MDA (and similar compounds) produce a colored-fluorescent product that may be measured spectrophotometrically or fluorometrically (3). A standard curve is generated, and sample lipid peroxidation product concentrations are estimated from that curve.

KIT CONTENTS

- 1 Microtiter plate that may be used to measure either optical density or to determine relative fluorescence.
- 1 Vial of MDA Standard: Supplied at 300 µM MDA.
- 1 Bottle of Assay Diluent: This must be reconstituted with 100 mL of purified water prior to use.
- 1 Bottle of TBA Reaction Buffer: This is 50% Acetic Acid.
- 1 Bottle of TBA Reagent: 2.5 mL of this reagent is added to the bottle of TBA Reaction Buffer, and mixed together to make the TBA Working Reagent.

Materials required, but not supplied

- Reaction tubes: Reaction tube used depends on the assay volume. This assay is compatible with either a 2.5 mL assay volume (for standard cuvettes) or a 0.5 mL assay volume (for supplied microtiter plate).
  - 16 x 120 mm screw-cap glass tubes for 2.5 mL reaction volumes.
  - 2.0 mL screw-cap microcentrifuge tubes for 0.5 mL reaction volumes
  - Appropriate tube supports
- Water bath or heat block set at 75 °C
- Graduated cylinders
- Purified water
- Disposable gloves
- Disposable pipettes
- Pipettes or pipettors depending on final assay volume
- Disposable cuvettes for conventional spectrophotometers or fluorometers

Detection device:

This kit is compatible with any of the following.

- UV-Vis Spectrophotomer
- Fluorometer
- Plate reader for spectrophotometry (capable of reading optical density (OD) of 532 nm)
- Fluorometric microplate reader: Ex 532 nm, Em 552 nm

Sample Collection and Storage

1. Store all kit reagents at 2-8 °C. The components should be used before the expiration date indicated on the outside of the box.
2. Serum and urine samples can be evaluated with this kit.
3. Clarify samples by centrifugation if necessary. Turbidity must be eliminated to produce accurate results.
4. Other sample types may require further processing and/or optimization by the user.

PROCEDURE

Place all reagents on the lab bench and allow them to come to room temperature.

1. Add 100 mL of purified water to the Assay Diluent Bottle to reconstitute this buffer. It will be 1X PBS.
2. Prepare all dilutions in Assay Buffer. For standards, complete the serial dilution of MDA in tubes leaving behind the required assay volume (this is addressed further below).

Completing the assay using 2.5 mL volumes destined for standard cuvettes

1.0 and 5.0 mL disposable pipettes are useful for this procedure.

1. Set up a water bath at 75 °C.
2. Secure a supply of 16 x 120 mm glass screw cap tubes.
3. Label 11 tubes as follows: C (control), 1-10.
4. Transfer 0.5 mL of Assay Diluent into each tube.
5. Place the C tube in another tube support.
6. Vortex the MDA Standard briefly.
7. Using a 1.0 mL pipette, transfer 0.5 mL of standard to tube 1.
8. Mix by pipetting up and down 5 times.
9. Using the same pipette: Transfer 0.5 mL from tube 1 to tube 2 (move tube 1 to the other support with the C tube).
10. Mix as before.
11. Continue to serially dilute the standard through tube 10 (moving tubes to the other support).
12. Remove and discard 0.5 mL from the last dilution in tube 10.
13. Now there is a control and standard diluted from 150 µM to 0.293 µM MDA; each tube should have 0.5 mL.
14. Label new tubes appropriately, and dilute samples in a similar manner. It is wise to run a few dilutions of each sample in the assay.
15. Be sure to leave 0.5 mL in each tube.

Prepare the TBA working solution

Be sure that the TBA reagent is in liquid form. If necessary, warm it slightly to achieve solution.

1. Use a 5 mL disposable pipette to transfer 2.5 mL of TBA Reagent to the TBA Reaction Buffer bottle.
2. Replace and secure the cap.
3. Mix the contents of the bottle thoroughly.

Add the TBA Working Solution

1. Using a 5 mL disposable pipette, add 2.0 mL of TBA Working Reagent to each reaction tube.
2. Install and secure the screw caps.
3. Vortex each tube briefly.

Incubate the tubes at 75 °C

1. Place the support containing the tubes directly into the water bath.
2. Be sure the temperature is at least 75 °C (slightly higher temperature is fine, up to 90 °C).
3. Leave in place for at least 3 hours.
4. Remove from water bath and allow to cool to RT.
5. Vortex each tube briefly.

Measuring the OD: Using standard sized disposable cuvettes

1. Use a 2 mL pipette to transfer 2.0 mL from C tube to a cuvette.
2. Use a fresh 2 mL pipette, and transfer 2 mL from tube 1 to a second cuvette.
3. With a fresh pipette for each reaction tube, continue preparing cuvettes with the diluted standard and samples.
4. Set up spectrophotometer for 532 nm.
5. Use a purified water as a reference (in dual beam instruments), and "blank" the spectrophotometer against the "C" tube cuvette.
6. Then determine the ODs for the remaining samples.

Determining the Relative Fluorescence: Fill disposable fluorimetry cuvettes as above

1. Set excitation wavelength to 532 nm.
2. Set emission wavelength to 553 nm.
3. "Blank" the machine against the "C" cuvette.
4. Place cuvette "4" in sample compartment: This will probably give the highest reading as higher concentration MDA results in quenching of signal. Set the sensitivity of the instrument based on this sample.
5. Determine RF for all standard dilutions and sample ones.

Analyze the data: The OD and RF standard curves show a linear relationship between MDA concentration and absorbance or RF.

Completing the assay using 0.5 mL volumes

Use 2.0 mL screw-cap microcentrifuge tubes. A 5 mL disposable pipette is needed to prepare the TBA Working Reagent. Micropipettes and tips capable of delivering 100, 400, and 500 µL are useful for this procedure.

1. Set-up dry bath/heating block to 75 °C.
2. Prepare tubes labeling "C" and 1-10 as above.
3. Transfer 0.1 mL of Assay Diluent into each tube using a pipettor set to 100 µL.
4. Move the C tube to another rack.
5. Add 100 µL of MDA Standard to tube 1. Mix thoroughly.
6. Serially dilute the standard as above, but use 100 µL transfers.
7. Remove and discard 100 µL from tube 10.
8. Dilute samples again leaving 100 µL in each tube.

Add TBA Working Reagent

Prepare the TBA Working reagent by adding 2.5 mL of TBA Reagent to the TBA Reaction Buffer Bottle. Mix thoroughly.

1. Add 400 µL of TBA Working Reagent to each tube.
2. Close the caps of the tubes.
3. Vortex briefly.

Place in heating block

1. Incubate at 75 °C for three hours.
2. Remove tubes and allow to cool to RT.
3. Vortex briefly.

Transfer to microplate

1. Prepare a plate map.
2. Transfer 200 µL of the "C" tube to the "blank" well.
3. Using a fresh tip for each reaction tube, transfer 200 µL volumes to the plate for each one.

Determine OD or RF using a plate reader

DATA ANALYSIS

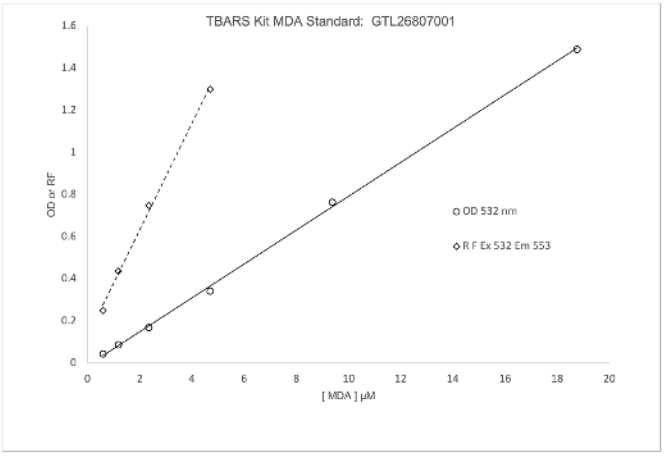
OD (RF) show linear response to concentration within the usable portion of the standard curve.

The data may be analyzed using a linear model, and unknowns may be determined accordingly. The calculations are easily completed with a spreadsheet program such as Excel®.

LIMITATIONS

1. Hemolyzed, icteric or grossly lipemic samples may not be suitable for analysis in this assay.
2. Turbid samples should be clarified before analysis.
3. If turbidity occurs after the incubation, try clarifying by centrifugation before determining the OD or RF.

This assay is run in 50% Acetic Acid. The TBA reaction occurs with numerous substances, and efforts to make it specific to MDA include protein removal, attention to pH and extraction of the TBA/MDA adduct. Users should review the references to determine suitability.



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2. Abeyrathne, EDNS, Nam, K, and DU Ahn. 2021. Analytical methods for lipid oxidation and antioxidant capacity in food systems. *Antioxidants 10*(10): 1587. https://doi.org/10.3390/antiox10101587
3. K Yagi. 1998. Simple method for the level of total lipid peroxides in serum or plasma. *Methods in Mol Biol* 108: 101-106.
4. H Ohkawa et. al. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95:351-358.
5. K Yagi. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* 15:212-216.

PRODUCT INFORMATION

CAT. #	DESCRIPTION
1020	TBARS Malondialdehyde Oxidative Stress Assay

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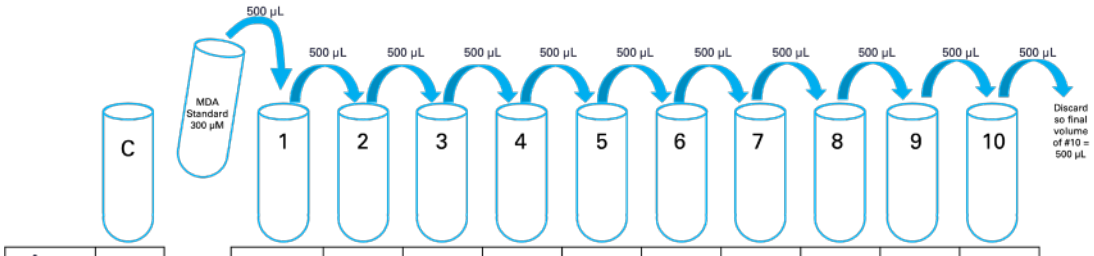
TRADEMARKS

APPENDIX

QUICK REFERENCE TEST PROCEDURE FOR TBARS ASSAY

Procedure for 2.5 mL volume (standard cuvette spectrophotometer or fluorometer instrumentation).

1. Warm reagents to room temperature.
2. Prepare Assay Diluent: Add 100 mL purified water to Assay Diluent bottle. Mix thoroughly.
3. Label tubes.
4. Prepare Control and MDA Standards:



Assay Diluent (µL)	500	500	500	500	500	500	500	500	500	500
MDA Standard	0	500 µL of Standard	500 µL of 1	500 µL of 2	500 µL of 3	500 µL of 4	500 µL of 5	500 µL of 6	500 µL of 7	500 µL of 8	500 µL of 9
Final conc. of MDA (µM)	0	150	75	37.5	18.75	9.38	4.69	2.34	1.17	0.59	0.29

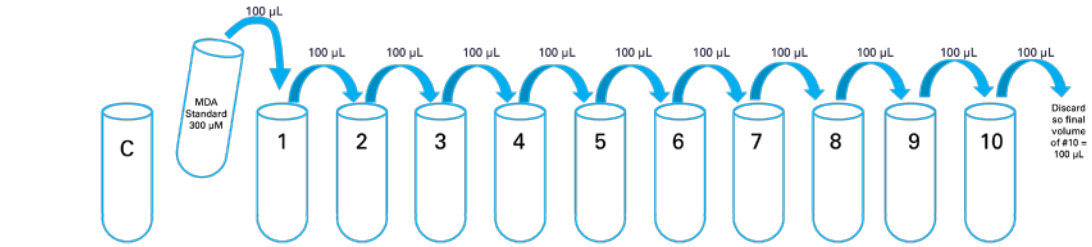
5. Prepare your samples to the appropriate dilution using prepared Assay Diluent.
6. Ensure each tube contains 500 µL.
7. Prepare TBA Working Reagent: Add 2.5 mL TBA Reagent to the TBA Reaction Buffer Bottle.
8. Add 2.0 mL TBA Working Reagent to each tube.
9. Tighten caps.
10. Heat in water bath set at 75 – 90 °C.
11. Cool to room temperature.
12. Transfer 2.0 mL from each tube to a disposable cuvette.
13. Determine OD at 532 nm or relative fluorescence (Ex 532/Em 553 nm).
14. Calculate concentration of TBA reactive substances using the linear portion of the MDA standard curve.

APPENDIX

QUICK REFERENCE TEST PROCEDURE FOR TBARS ASSAY, CON'T

Procedure for 0.5 mL volume (96-well plate reader).

1. Warm reagents to room temperature.
2. Prepare Assay Diluent: Add 100 mL purified water to Assay Diluent bottle. Mix thoroughly.
3. Label tubes.
4. Prepare Control and MDA Standards:



Assay Diluent (µL)	100	100	100	100	100	100	100	100	100	100
MDA Standard	0	100 µL of Standard	100 µL of 1	100 µL of 2	100 µL of 3	100 µL of 4	100 µL of 5	100 µL of 6	100 µL of 7	100 µL of 8	100 µL of 9
Final conc. of MDA (µM)	0	150	75	37.5	18.75	9.38	4.69	2.34	1.17	0.59	0.29

5. Prepare your samples to the appropriate dilution using prepared Assay Diluent.
6. Ensure each tube contains 100 µL.
7. Prepare TBA Working Reagent: Add 2.5 mL TBA Reagent to the TBA Reaction Buffer Bottle.
8. Add 400 µL TBA Working Reagent to each tube.
9. Tighten caps.
10. Heat in water bath set at 75 – 90 °C.
11. Cool to room temperature.
12. Transfer 200 µL from each tube to the supplied 96-well microplate.
13. Determine OD at 532 nm or relative fluorescence (Ex 532/Em 553 nm).
14. Calculate concentration of TBA reactive substances using the linear portion of the MDA standard curve.

APPENDIX

TBARS Assay					Date:	
Experiment#						
Technician:						
Reagent			**P/N**		**L/N**	
TBARS Assay Plate (Microtiter plate)			1TB0106			
MDA Standard (300 µM)			1TB0101			
TBARS Assay Diluent (10X PBS)			1TB0216			
TBA Reagent Solution in DMSO			1TB0400			
TBA Reaction Buffer			1TB0200			
TBARS Assay Diluent Preparation: Add 100 mL of purified water to TBARS Assay Diluent Bottle. Mix Well TBA Working Reagent: Prepare immediately before use: Add 2.5 mL TBA Stock to the TBA Reaction Buffer bottle. Mix Well						
Tube #	**Contents**	**1/Dilution**	**[ ]: µM**	**Comment**		
C	CONTROL: TBA DILUENT					
1	MDA	2	150.000			
2	MDA	4	75.000			
3	MDA	8	37.500			
4	MDA	16	18.750			
5	MDA	32	9.375			
6	MDA	64	4.688			
7	MDA	128	2.344			
8	MDA	256	1.172			
9	MDA	512	0.586			
10	MDA	1024	0.293			
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APPENDIX

Continued						
Tube #	Contents	1/Dilution	[ ]: µM	Comment		
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