INTENDED USE

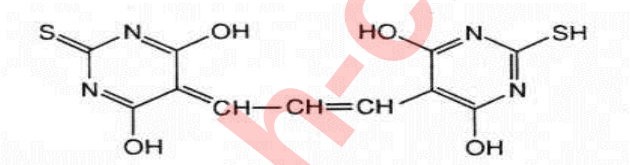
The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress [1-3]. This rapid, easy-to-use procedure has been modified by researchers for use with many types of samples including drugs, food products and human and animal biological tissues [4-7]. The assay has provided important information regarding free radical activity in disease states and has been used for measurement of anti-oxidant activity of several compounds [8-9]. Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than MDA, it remains the most widely employed assay used to determine lipid peroxidation [10]. If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized. Nonetheless, if TBARS are increased, it is recommended that a more specific assay such as HPLC be performed.

The OXItek TBARS Kit is designed to provide a standardized, reproducible assay with consistent results. Each lot of reagents is quality controlled as a kit, which includes an MDA standard. It is recommended that additional in-house controls are included in each test run.

The OXItek TBARS Assay Kit is for Research Purposes Only.

PRINCIPLE OF THE SYSTEM

Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid and produces the following:



which can be measured by fluorometry or spectrophotometry. Although this reaction has a much higher sensitivity when measured via fluorometry, protocols for both methods are provided in the Test Procedure section of this insert.

Biological specimens contain a mixture of thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS return to normal levels over time, depending upon the presence of anti-oxidants. In practice, TBARS are expressed in terms of malondialdehyde (MDA) equivalents [11]. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted.

The OXItek TBARS Assay Kit provides all the necessary reagents to perform 160 tests and is designed for researchers studying oxidative stress and anti-oxidant activity. It is recommended that in-house controls be run with each sample test.

Depending on geographic location, normal plasma and serum TBARS should be <1.5 and <2.0 MDA units respectively. Mean and ± SD or SE must be established by each laboratory. It is recommended that samples be run in duplicate.

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Limitation to Procedure:

1. Hemolyzed, icteric or grossly lipemic plasma samples are not suitable for use in TBARS analysis.
2. Non-lipid TBARS may be present in the sample. It is recommended that a sample with elevated TBARS levels be tested by a more specific test for lipid peroxidation such as HPLC.
3. Normal tissues contain very low levels of free malondialdehyde.

REAGENTS

Materials Supplied:

- **Thiobarbituric Acid (4 vials/kit):** Contains 0.53 grams thiobarbituric acid
- **TBARS Diluent 1 (4 x 50 mL/kit):** Contains acetic acid
- **TBARS Diluent 2 (4 x 50 mL/kit):** Contains sodium hydroxide
- **SDS Solution (30 mL/kit):** Contains sodium dodecyl sulfate
- **MDA Standard (20 mL/kit):** Contains 100 nmol/mL Malondialdehyde Bis (dimethyl acetal)
- **MDA Diluent (100 mL/kit):** Contains sterile deionized water
- **Marbles**

Storage:

Store all kit reagents at 2-8˚C. The components should be used before the expiration date indicated on the outside of the box.

Materials Required but not Supplied:

- 12 x 75 mm glass test tubes and metal racks
- Disposable gloves
- Adjustable pipettes
- Graduated cylinders and assorted beakers
- Stir plate
- Heat block, incubator or water bath set at 95˚C
- Fluorometer or Spectrophotometer

PRECAUTIONS

- To avoid cross contamination, use separate pipette tips for each specimen.
- TBARS Diluent 1 contains acetic acid. Handle and dispose of according to applicable legal and safety guidelines.
- TBARS Diluent 2 contains sodium hydroxide. Handle and dispose of according to applicable legal and safety guidelines.
- If reusing glass tubes, be certain to rinse thoroughly in deionized water.

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PREPARATION OF REAGENTS

Note: Prepare fresh for each analysis.

TBA/Buffer Reagent:

Use 1 bottle of TBARS Diluent 1 (50 mL), 1 bottle of TBARS Diluent 2 (50 mL) and 1 vial of Thiobarbituric Acid (TBA). One hundred mL is sufficient for approximately 40 tubes.

Add TBA to a mixing vessel containing half a bottle of TBARS Diluent 1. Rinse the vial with the remaining half of TBARS Diluent 1 and add to the mixing vessel. Add a full bottle of TBARS Diluent 2. Mix until the TBA is completely dissolved. If necessary, very low heat may be used.

MDA Standard for Fluorometer:

Dilute MDA Standard 1:10 by mixing 1.8 mL of MDA Diluent and 0.2 mL of MDA Standard. Mix thoroughly. Prepare a series of 5 standards in MDA Diluent. The dilution scheme for these standards is given below in Table 1.

Table 1: Preparation of MDA Standard for Fluorometer

Standard Number	Concentration of MDA	Volume of 1:10 MDA Standard	Volume of Diluent
4	4 nmol/mL	400 µL	600 µL
3	3 nmol/mL	300 µL	700 µL
2	2 nmol/mL	200 µL	800 µL
1	1 nmol/mL	100 µL	900 µL
0	0 nmol/mL	0 µL	1000 µL

* The concentration of the 1:10 standard is 10 nmol/mL, and may be used directly as an additional standard when assaying samples expected to have elevated TBARS, such as urines.

MDA Standard for Spectrophotometer:

Use MDA Standard undiluted for 100 nmol/mL standard. Prepare a series of 5 standards in MDA Standard Diluent. The dilution scheme for these standards is given below in Table 2.

Table 2: Preparation of MDA Standard for Spectrophotometer

Standard Number	Concentration of MDA	Volume of MDA Standard	Volume of Diluent
4	100 nmol/mL	1000 µL	0 µL
3	50 nmol/mL	500 µL	500 µL
2	25 nmol/mL	250 µL	750 µL
1	12.5 nmol/mL	125 µL	875 µL
0	0 nmol/mL	0 µL	1000 µL

TEST PROCEDURE

Allow all reagents to reach room temperature before use. SDS Solution will take at least one hour if stored at 2-8°C. Heating the SDS Solution at 37˚C briefly will redissolve precipitated SDS. SDS Solution can then be stored at room temperature.

Step 1: Collect EDTA plasma (lavender top Vacutainer®). For preparation of other sample types, see sample preparation section of this insert.

Step 2: Label each disposable glass test tube with the standard number or sample identification.

Step 3: Add 100 µL sample or standard to properly labeled tube.

Step 4: Add 100 µL SDS Solution to each tube and swirl to mix.

Step 5: Add 2.5 mL TBA/Buffer Reagent forcefully down the side of each tube.

Step 6: Cover each tube with a glass marble and incubate at 95˚C for 60 minutes.

Step 7: Remove from incubation and cool to room temperature in an ice bath for 10 minutes.

Step 8: Centrifuge samples at 1200 x g for 15 minutes.

Step 9: Remove supernatant from samples for analysis.

Step 10: *Fluorescent Analysis:* read supernatants with excitation set at 530 nm and emission at 550 nm. It is recommended sensitivity be set at high with a slit width of 5 nm.
Spectrophotometer Analysis: Read absorbance of supernatants at 532 nm.

SAMPLE PREPARATION

Note: Samples should be tested immediately. If serum/plasma samples are not tested immediately, remove from clot, aliquot and store at -70°C.

Platelets:

Collect 5 mL specimen of heparinized venous blood (green top Vacutainer®) from patients who have fasted overnight. Remove stopper and let RBCs sediment by gravity. Pipet off platelet-rich plasma (PRP) into clean, plastic centrifuge tube after 1 mL has formed and continue until separation is complete. Centrifuge PRP at 300 x g for 5 min, collect supernatant and centrifuge the supernatant at 2000 x g for 15 minutes to sediment platelets.

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Leukocytes:

Collect blood in EDTA (lavender top Vacutainer®). Allow RBCs to sediment; collect upper "Buffy Coat". Wash leukocytes contained in the Buffy Coat once in an isotonic solution such as saline or Dulbecco's PBS. Resuspend leukocytes in isotonic solution for testing [15].

Erythrocyte Ghosts:

Wash RBC pellet from leukocyte separation with 20 mL of TRIS Buffer, pH 7.4 (6.05 g TRIS, 6.42 g NaCl, 420 mL 0.1M HCl, 580 mL deionized water), centrifuge at 1600 x g for 10 minutes, discard supernatant and repeat twice. Add an equal volume of TRIS Buffer to final pellet and incubate a minimum of 4 hours at 4°C. Lysis of erythrocytes is performed on ice with pre-cooled conditions. Add 15 mL lysing solution (301 mg $MgSO_4$, 372 mg KCl in 500 mL of sterile water) to 0.5 mL of the cell suspension. Immediately add 1 mL of resealing solution (53.7 g KCl, 10.5 g NaCl in 400 mL of deionized water). Keep the suspension on ice for 5 minutes and then at 37°C for 30 minutes. Pellet the ghosts by centrifuging for 10 minutes at 3000 x g [12].

Oxidized LDL:

Plasma: Collect fasting heparinized whole blood. Centrifuge at 1600 x g for 10 minutes at 5-10°C, carefully remove plasma and place on ice for immediate analysis or freeze several aliquots at -70°C for later analysis. Samples can be safely stored for 1-2 months. Process as described below for serum [3].

Serum: Collect fasting whole blood in a red top vacutainer®. Incubate at room temperature for at least 30 minutes for clots to form. Centrifuge at 1600 x g for 10 minutes. Carefully remove serum and place on ice for immediate analysis or freeze aliquots at -70°C for later analysis. Add 1.5 mL of isotonic saline to 1.5 mL of plasma/serum. Prepare a sodium heparin solution in water containing 10,000 USP units per mL. Prepare a solution of 1.3M manganese chloride in water. A working reagent is prepared with 1.2 mL sodium heparin solution to 4.1 mL manganese chloride solution. Scale up as necessary. Add 300 µL of working reagent to the diluted serum/plasma. Vortex and allow the LDL/VLDL to precipitate for 15 minutes at room temperature. Centrifuge the serum/plasma at 10,000 x g for 15 minutes. Decant the serum/plasma supernatant and resuspend the pellet in 1.5 mL normal saline or PBS [3,13]. If required, sonicate each sample 5 seconds at low setting over ice. Use these samples to start the TBARS assay, substituting water for SDS in the reaction mixture.

Note: When analyzing samples that may be non-fasting, triglycerides measuring above 300-350 mg/dL will yield a turbid supernatant and cannot be measured.

Other Body Fluids:

The TBARS kit is suitable for analysis of urine, CSF, vaginal, synovial, seminal, vitreous, tears, saliva, sperm, pulmonary and gastrointestinal fluids and lavages.

Tissues:

Freeze tissue in liquid nitrogen and immediately crush in a pre-chilled mortar and pestle. Resuspend tissue at 50 mg/mL in normal saline or PBS. Disrupt in a Potter-Elvejhem glass homogenizer. If necessary, sonicate for 15 seconds at 40V setting over ice and use uncentrifuged whole homogenate for analysis. Alternatively, homogenize in isotonic media appropriate for sub-cellular fractionation to study TBARS in plasma membranes, nuclear membranes or organelles. Centrifuge at 1600 x g for 10 minutes at 4ºC. Supernatants may be used for enzyme analyses. Recommend normalizing TBARS values to another constituent such as protein.

Cell Cultures:

Suspend 20 million cells in 1 mL of cell culture medium or buffer of choice such as PBS. Sonicate for 5 second intervals at 40 V setting over ice. Use whole homogenates in the assay, being sure to use culture fluid as a sample blank.

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Anti-Oxidant Screening:

The TBARS assay may be used for testing anti-oxidants and drugs [13].

Oxidize 2 100 µL aliquots of a sample, such as serum or plasma, with 5mM ferric chloride. Add the anti-oxidant compound to be tested to one. Incubate plasma alone plus the 2 test samples at 37°C for 30 minutes. Run the TBARS assay and compare to a distilled water control. Calculate percentage of inhibition [14]. Drugs may be added directly to plasma samples and compared to plasma alone in the TBARS assay.

CALCULATION AND INTERPRETATION OF RESULTS

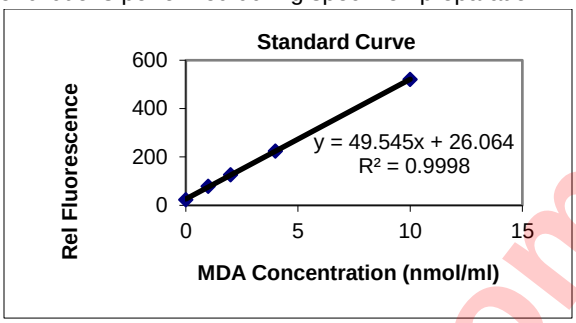
Typical Standard Curve (Fluorometer):

This is an example of a typical standard curve and is not to be used for interpretation of results. Variation may occur in individual laboratories due to pipetting, laboratory and incubator temperatures, etc.

Table 3: Sample Standard Curve

MDA	Fluorometer Readings
10 nmol/mL	521.15
4 nmol/mL	223.68
2 nmol/mL	126.48
1 nmol/mL	78.69
0 nmol/mL	22.5

For Fluorometer:
Using linear graph paper, plot mean MDA equivalents for each standard used on the X-axis versus the corresponding fluorometer reading on the Y-axis. Determine the concentration of MDA equivalents in nmol/mL in specimens by interpolation from the standard curve. Correct sample values for any other dilutions performed during specimen preparation.



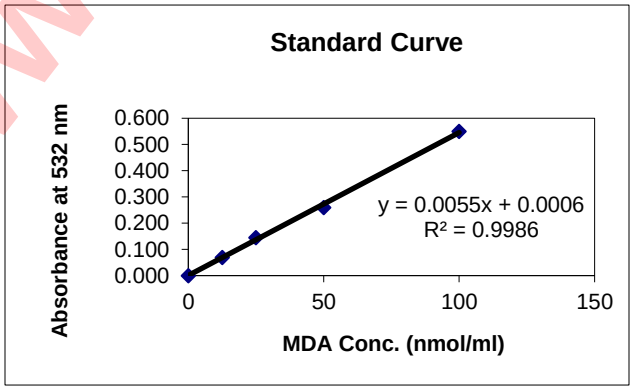
Graph 1

Typical Standard Curve (Spectrophotometer):
This is an example of a typical standard curve and is not to be used for interpretation of results. Variation may occur in individual laboratories due to pipetting, laboratory and incubator temperatures, etc.

Table 4: Sample Standard Curve

MDA Concentration	Spectrophotometer Readings
100 nmol/mL	0.550
50 nmol/mL	0.260
25 nmol/mL	0.145
12.5 nmol/mL	0.070
0 nmol/mL	0.000

For Spectrophotometer:
Using linear graph paper, plot mean MDA equivalents for each standard used on the X-axis versus the corresponding spectrophotometer reading on the Y-axis. Determine the concentration of MDA equivalents in nmol/mL in specimens by interpolation from the standard curve. Correct sample values for any other dilutions performed during specimen preparation.



Graph 2

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PROCEDURAL FLOW CHART

PREPARE REAGENTS
↓
PIPET SPECIMENS AND CONTROLS
↓
ADD SDS SOLUTION
↓
ADD TBA/BUFFER REAGENT
↓
INCUBATE 60 MINUTES AT 95˚ ±1˚C
↓
COOL TO ROOM TEMPERATURE
↓
CENTRIFUGE SPECIMEN
↓
READ RESULTS

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