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100 Test Cuvette Assay for

2-Thiobarbituric Acid Reactive Substances (TBARS)

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INTRODUCTION

2-**T**hio**B**arbituric **A**cid **R**eactive **S**ubstances (TBARS) are naturally present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress.[1,2] TBARS assay values are usually reported in malonaldehyde (malondialdehyde, MDA) equivalents, a compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides. The TBARS assay is a well-recognized, established method for quantifying these lipid peroxides, although it has been criticized for its reactivity towards other compounds other than MDA.[3] This kit offers the researcher a straightforward, reproducible and consistent method for analyzing urine for lipid peroxidation products.

PRINCIPLES OF PROCEDURE

This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25°C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid *via* a Knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm, as shown below in Figure 1:

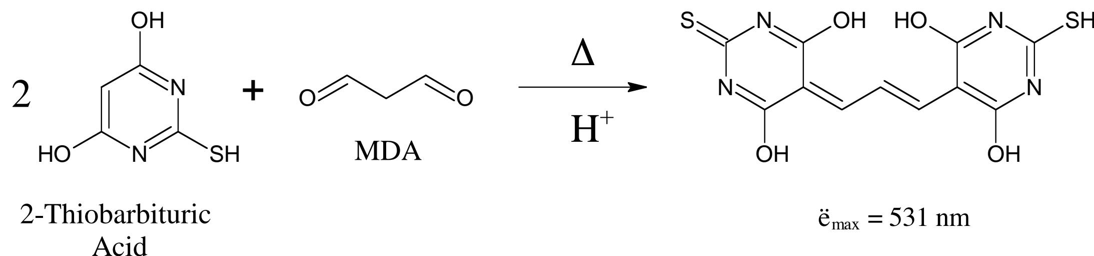


Figure 1. Reaction between 2-thiobarbituric acid and MDA under acidic conditions.

MATERIALS PROVIDED

Component	Description	Amount	Storage	Cat. No.
Indicator	2-Thiobarbituric Acid	4 x 3.0 g	4°C	FR35a
Acid Reagent	10% Acid Solution in Dimethylsulfoxide	125 mL	4°C	FR35b
MDA Standard	10 mM Malonaldehyde Tetrabutylammonium Salt	100 μL	4°C	FR35c

MATERIALS NEEDED BUT NOT PROVIDED

1. Spectrophotometer set to 532 nm (540 nm may also be used)
2. Glass test tubes
3. Deionized Water (dH_2O)
4. Adjustable micropipettes (10 – 1,000 μL) and tips
5. Spectrophotometric cuvettes (0.5 mL or 1 mL volume)

STORAGE

1. The reagents are stable until the indicated kit expiration date if handled and stored properly.
2. When not in use, store the kit at 4°C for up to one year.
3. MDA standards should be used within 24 hours of preparation.
4. The Indicator Solution (combined 2-TBA and Acid Reagent) can be stored at 4˚C for one week.

WARNINGS AND PRECAUTIONS

1. Use aseptic techniques when opening and dispensing reagents.
2. Wear gloves and safety glasses when performing this assay, as the acid used is corrosive.
3. In case of accidental exposure to 2-TBA or Acid Reagent, thoroughly wash the exposed area with soap and water.
4. 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

1. Do not leave the reagent bottles open. Replace the caps as soon as the desired volume is removed.
2. To minimize error due to handling, wipe the exterior of the cuvettes with a lint-free paper towel.
3. **Sample Blanks:** It is HIGHLY recommended that, for each sample analyzed, a sample blank is also performed to account for background interferences. While Oxford's TBARS method has the lowest background of all commercial assays, some background interferences are inherent in biological samples and cannot be avoided. The sample blank should contain the sample plus the Acid Reagent alone without TBA indicator. Following this procedure will result in more accurate, precise and reliable TBARS measurements.
4. There are sufficient reagents for 100 tests.

REAGENT PREPARATION

1. **Acid Reagent:** This solution may need to be thawed upon removal from 4°C. Allow to sit at room temperature for one hour. It is ready to use once thawed.
2. **Indicator Solution:** Add 30 mL of the Acid Reagent to the powdered contents of one bottle of Indicator and shake until completely dissolved. One bottle is sufficient for 25 tests.
3. **20 μM MDA Standard Stock (Colorimetric):** Dilute the 10 mM MDA Standard 1:500 in dH_2O by adding 20 μL of 10 mM MDA to 9.98 mL dH_2O. Prepare immediately prior to use.
4. **20 nM MDA Standard Stock (Fluorometric):** Further dilute the 20 μM MDA Standard Stock solution from above 1:1,000 in dH_2O by adding 10 μL to 9.99 mL dH_2O. Prepare immediately prior to use.

SAMPLE STABILITY

Studies at OBR show that this kit provides optimum results with urine that is less than 48 hours old, and is preferably run immediately after sample collection. If this is not possible, samples should be frozen at -70°C to prevent loss of MDA and HAE[4] and sample oxidation. Samples should **not** be stored at -20°C. Samples should not be refrozen and should be protected from light to avoid photooxidation.

SAMPLE PREPARATION

When working with plasma, the sample should be deproteinated with an acid. Centrifuge and use the supernatant to perform the assay. This solution may appear cloudy after the reaction, and can be clarified by passing through a 0.2 μ syringe filter.

When working with urine, colored compounds contribute to the signal measured at 532 nm. This interference can be removed by running a sample blank with each sample.

Urine

1. Urine samples can be used directly and should be assayed immediately. If the assay is to be performed on a different day, the sample should be stored at -70°C.

Plasma

1. Collect blood with an additive such as heparin, EDTA or citrate to prevent coagulation.
2. Centrifuge the sample for 10 minutes at 2000 × g at 4°C.
3. Carefully remove the straw-colored plasma layer and store on ice for use on the same day, or at -70°C for up to thirty days.
4. Plasma samples can be run without dilution.

Serum

1. Collect whole blood without the addition of any additives, such as an anticoagulant.
2. At room temperature, allow the blood to clot for 30 minutes.
3. Centrifuge the sample at 2,000 × g for 15 minutes at 4°C.
4. Carefully remove the straw-colored serum layer and store on ice for use on the same day, or at -70°C for up to thirty days.
5. Serum samples can be run without dilution.

STANDARD CURVE PREPARATION

Malondialdehyde is provided as a solution of the malondialdehyde tetrabutylammonium (MDA-TBA) salt in a slightly basic buffer because MDA itself is not stable. When mixed with the acidic Indicator Solution, the MDA-TBA molecule is acidified and generates MDA quantitatively.

Please see the **Reagent Preparation** section for preparing the 20 μM MDA Standard Stock.

Table 1: Colorimetric Standard Curve Preparation

Standard	MDA Conc. (μM)	Vol. of dH_2O (μL)	Vol. of 20 μM MDA Stock (μL)
S_0	0	1000	-
S_1	0.5	975	25
S_2	1.0	950	50
S_3	2.5	875	125
S_4	5.0	750	250
S_5	10.0	500	500
S_6	15.0	250	750
S_7	20.0	-	1000

ASSAY PROCEDURE

Free MDA

1. Preparation of Standards and Samples: Add each of the following reagents into glass test tubes and mix well.
   - **Standards**: 0.4 mL of standard and 1.2 mL of Indicator Solution.
   - **Samples**: 0.4 mL of sample and 1.2 mL of Indicator Solution.
   - **Blanks**: 0.4 mL of sample and 1.2 mL of Acid Reagent.
2. After the standards, samples and blanks have been mixed; allow them to react for 20 minutes at room temperature.
3. Transfer 1.5 mL of each solution to cuvettes and read at 532 nm. The pink color is stable for several hours at room temperature.

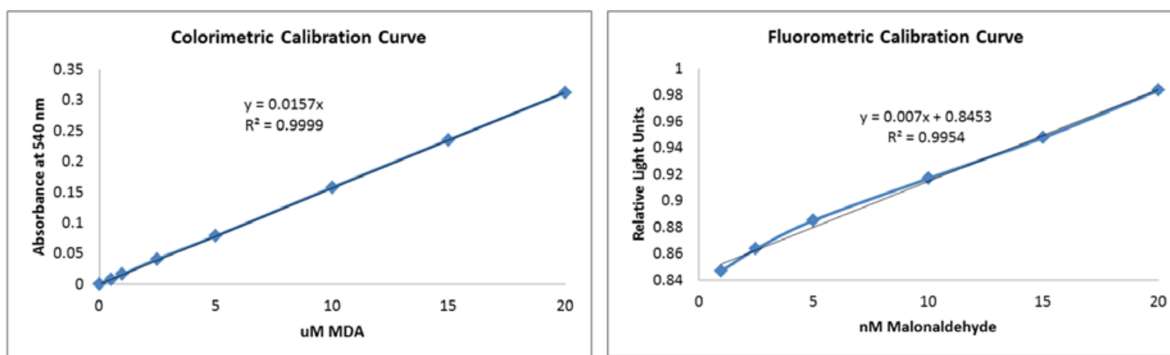
Total MDA

1. Prepare samples and standards exactly as above, but heat sample at 65˚C for 30 minutes, then follow step 3 as above.

CALCULATIONS

1. Plot a standard curve using the A_{532} OD value for each Standard versus the MDA concentration for each Standard. The equation of the line can be found using a linear fit method.
2. Subtract the OD (A_{532}) for each Sample Blank from the Sample OD (A_{532}) to obtain a Net OD (A_{532}).
3. Calculate the MDA concentration for each Sample using the Net OD (A_{532}) value and the equation generated by the Standard Curve. If the Samples were diluted, the result must be multiplied by the dilution factor.

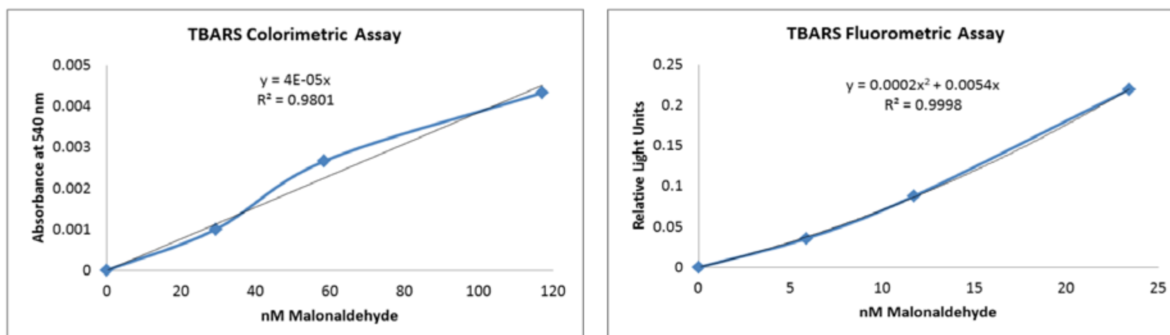
Figure 2: Typical Standard Curves



LIMITS OF DETECTION

The limits of detection have been determined to be 250 μM and 5.0 nM for the colorimetric and fluorometric assays, respectivly.

Figure 3: Limits of Detection



PERFORMANCE LIMITATIONS

1. Although the standards in this assay will usually appear water clear, the samples may become colored. This is due to the formation of additional chromophores that absorb at various wavelengths other than 532 nm and will usually not interfere with the A_{532} signal.
2. In setting up this assay for the first time on a particular biological sample, the kinetics of color development in the samples should be followed in comparison with those of the MDA standards. The A_{532} of the standards should reach a plateau after approximately 15 minutes and then remain. If the A_{532} signal continues to increase after the standards have achieved a stable color, the researcher should be concerned that interfering non-TBARS related reactions are occurring in the sample.

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