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Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay

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Abstract

Despite its limited analytical specificity and ruggedness, the thiobarbituric acid reactive substances (TBARS) assay has been widely used as a generic metric of lipid peroxidation in biological fluids. It is often considered a good indicator of the levels of oxidative stress within a biological sample, provided that the sample has been properly handled and stored. The assay involves the reaction of lipid peroxidation products, primarily malondialdehyde (MDA), with thiobarbituric acid (TBA), which leads to the formation of MDA-TBA_2 adducts called TBARS. TBARS yields a red-pink color that can be measured spectrophotometrically at 532 nm. The TBARS assay is performed under acidic conditions (pH = 4) and at 95 °C. Pure MDA is unstable, but these conditions allow the release of MDA from MDA bis(dimethyl acetal), which is used as the analytical standard in this method. The TBARS assay is a straightforward method that can be completed in about 2 h. Preparation of assay reagents are described in detail here. Budget-conscious researchers can use these reagents for multiple experiments at a low cost rather than buying an expensive TBARS assay kit that only permits construction of a single standard curve (and thus can only be used for one experiment). The applicability of this TBARS assay is shown in human serum, low density lipoproteins, and cell lysates. The assay is consistent and reproducible, and limits of detection of 1.1 µM can be reached. Recommendations for the use and interpretation of the spectrophotometric TBARS assay are provided.

Keywords

Chemistry; Issue 159; thiobarbituric acid reactive substances; TBARS; oxidation; lipid peroxidation; malondialdehyde; MDA; thiobarbituric acid; TBA; oxidative stress

Introduction

Lipid peroxidation is a process in which free radicals, such as reactive oxygen species and reactive nitrogen species, attack carbon-carbon double bonds in lipids, a process that involves the abstraction of a hydrogen from a carbon and insertion of an oxygen molecule.

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This process leads to a mixture of complex products including, lipid peroxyl radicals, and hydroperoxides as the primary products, as well as malondialdehyde (MDA) and 4-hydroxynonenal as predominant secondary products[1].

MDA has been widely used in biomedical research as a marker of lipid peroxidation due to its facile reaction with thiobarbituric acid (TBA). The reaction leads to the formation of MDA-TBA_2, a conjugate that absorbs in the visible spectrum at 532 nm and produces a red-pink color[2]. Other molecules derived from lipid peroxidation besides MDA can also react with TBA and absorb light at 532 nm, contributing to the overall absorption signal that is measured. Similarly, MDA can react with most other major classes of biomolecules, potentially limiting its accessibility for reaction with TBA[3,4]. As such, this traditional assay is simply considered to measure "thiobarbituric acid reactive substances" or TBARS[5].

When correctly applied and interpreted, the TBARS assay is generally considered a good indicator of the overall levels of oxidative stress in a biological sample[6]. Unfortunately, as documented by Khoubnasabjafari and others, the TBARS assay is often conducted and interpreted in ways that facilitate dubious conclusions[3,4,7,8,9,10,11]. The causes for this are rooted primarily in sample-related pre-analytical variables and a lack of assay ruggedness that prohibits seemingly minor variations in assay protocol without substantial changes in assay results[1,7,12,13].

Preanalytical variables related to biospecimen handling and storage (e.g., blood plasma kept temporarily at –20 °C)[14,15] can have a major impact on TBARS assay results[16,17]; so much so, that TBARS assay results should not be compared across different laboratories unless warranted by explicit interlaboratory analytical validation data. This recommendation is akin to how western blots are commonly used and interpreted. Comparisons of band densities are valid for within-blot and perhaps within-laboratory studies, but comparing band densities between laboratories is generally considered an invalid practice.

Some researchers have suggested that MDA as measured by the TBARS assay simply does not meet the analytical or clinical criteria required of an acceptable biomarker[3,9,10,18,19]. Indeed, if the assay had not been developed over 50 years ago, it probably would not have gained the widespread use and tacit acceptability that it has today. Although there are other assays with greater analytical sensitivity, specificity, and ruggedness used for determining oxidative stress, TBARS assay based on absorbance at 532 nm remains by far one of the most commonly used assays for the determination of lipid peroxidation[20], and thereby assessment of oxidative stress.

The TBARS assay can only be found as an expensive kit (over 400 U.S. dollars), in which the instructions do not provide detailed information on most concentrations of the reagents used. Additionally, the reagents provided can only be used for one experiment, because only one colorimetric standard curve can be made per kit. This can be problematic for researchers who intend to determine levels of oxidation within a few samples at different timepoints, because the same standard curve cannot be used at multiple times. Hence, multiple kits need to be purchased for multiple experiments. Currently, unless an expensive kit is purchased, there is not a detailed protocol available for how to perform a TBARS

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assay. Some researchers in the past have vaguely described how to perform a TBARS assay[21,22], but neither a fully detailed protocol or comprehensive video on how to conduct the TBARS assay without an expensive kit is available in the literature.

Here we report a detailed, analytically validated for-purpose methodology on how to perform a TBARS assay in a simple, reproducible, and inexpensive way. Changes in the lipid peroxidation of human serum, HepG2 lysates, and low density lipoproteins upon treatment with Cu(II) ions are demonstrated as illustrative applications for the TBARS assay. Results demonstrate that this TBARS assay is consistent and reproducible on a day-to-day basis.

Protocol

Human serum specimens were obtained from consenting volunteers under IRB approval and according to the principles expressed in the Declaration of Helsinki. Specimens were coded and de-identified before transfer to the analytical laboratory.

1. Sample preparation

1. HepG2 cell lysates

1. Seed about 10×10^6 HepG2 cells per flask in 16 T75 flasks with 14 mL of EMEM media supplemented with 10% fetal bovine serum (FBS) and grow cells for 2 days.

2. Prepare RIPA buffer: in a 50 mL tube, add 1.5 mL of 5 M NaCl, 2.5 mL of 1 M Tris-HCl (pH = 7), 500 µL of NP-40 reagent, then bring the final volume to 50 mL with DI water.

3. Prepare lysis buffer: aliquot 20 mL of RIPA buffer into a 50 mL tube and add 200 µL of a 100x protease inhibitor solution to inhibit protein and lipid degradation. Store at 4 °C.

   NOTE: Lysis buffer is compatible with TBARS reagents and does not interfere with absorbance at 532 nm. If planning to use a different lysis buffer or add additional ingredients to the lysis buffer, preliminary validation studies need to be done to verify that lysis buffer components are compatible with the TBARS assay.

4. Remove media containing 10% FBS and wash cells 2x with 5 mL of cold, sterile 1x PBS.

5. Add 1 mL of lysis buffer to the T75 flasks containing the cells and incubate them for 10 min at room temperature (RT) with constant swirling to ensure the buffer is well-distributed.

6. Collect lysates into appropriately labeled 2 mL snap-cap polypropylene tubes and incubate on ice for 10 min.

7. Spin the lysates at 5,000 × g for 10 min at RT to collect cell debris, and aspirate supernatants into a single 15 mL tube.

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8. Concentrate cell lysate supernatant four-fold using a Speed Vac at 50 °C and 3 mbar and make aliquots of 94 µL each into 2 mL snap-cap polypropylene tubes. Store samples at −80 °C until they are used for in vitro oxidation and/or TBARS assay.

   NOTE: To avoid concentrating the cell lysate supernatant, cells can also be detached using 3 mL of 1x trypsin, neutralized with 6 mL of media, and washed 2x with 5 mL of cold PBS. Cell pellets can then be reconstituted in 250 µL of lysis buffer, and steps 1.1.6 and 1.1.7 can then be performed.

9. Prepare a 35 mM $CuCl_2$ stock solution in acetic acid (pH = 4).

   1. Prepare acetic acid solution (pH = 4): Dilute 1 µL of glacial acetic acid in 100 mL of DI water (pH should be approximately 4 but confirm this with a pH meter). Add more water or acetic acid to adjust the pH to 4.

   2. Weight out about 0.1936 g of copper II chloride and dissolve in 10 mL of the acetic acid solution (pH = 4) to make a 144 mM $CuCl_2$ stock. Aliquot 490 µL from this solution and add to 1,510 µL of acetic acid (pH = 4) to make a 35 mM $CuCl_2$ solution.

10. Aliquot 6 µL from the 35 mM $CuCl_2$ stock solution and add it to six samples containing 94 µL of cell lysate to make a final $CuCl_2$ concentration of about 2 mM. Add 6 µL of an acetic acid solution (pH = 4) that does not have $CuCl_2$ to six samples containing 94 µL of cell lysates to use as controls. The final volume of cell lysate should be 100 µL, which is what will be used for the TBARS assay.

    NOTE: Making the 35 mM $CuCl_2$ stock solution in acetic acid (pH = 4) is necessary to prevent precipitation of copper hydroxide.

11. Incubate samples in an oven at 37 °C for 24 h and perform a TBARS assay on each sample containing a final volume of 100 µL.

12. Repeat steps 1.1.9 and 1.1.11 2x on separate days to check the reproducibility of the TBARS assay for HepG2 cell lysates.

2. Low density lipoproteins—NOTE: Typically, pre-purified low density lipoprotein (LDL) contains some amount of EDTA. LDL samples used here contain 0.01% EDTA. EDTA can inhibit the in vitro Cu(II)-mediated oxidation of LDL. Hence, it may be necessary to remove EDTA from LDL samples prior to experiments or analysis. Steps 1.2.1–1.2.5 describe this process.

CAUTION: Sodium hydroxide is corrosive and causes irritation in skin and eyes. Use proper personal protective equipment.

1. Aliquot 24 µL from a 5.51 mg/mL LDL stock (protein concentration determined by modified Lowry method using BSA as a standard) into appropriately labeled 1 mL snap-cap polypropylene tubes. Make as many aliquots as needed and store at 4 °C until use in oxidation and/or TBARS assay.

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2. Prepare a 10 mM HEPES buffer in 0.15 M NaCl adjusted to pH = 7 with NaOH beads: dissolve 4.39 g of NaCl in 0.49 L of water, then add 1.19 g of HEPES. Dissolve well with a stir bar. Add sodium hydroxide beads until pH is 7. Dilute to 0.5 L with water. Store buffer at 4 °C and use within 3 months.

3. Add 476 μL of the 10 mM HEPES buffer in 0.15 M NaCl (pH = 7) to the aliquoted LDL samples to bring final volume to 500 μL. Add diluted LDL sample to a 0.5 mL centrifugal spin filter device with a 100K molecular weight cutoff.

4. Spin samples at 14,000 × g for 10 min at RT, leaving a final retentate volume of about 30 μL. Reconstitute samples in 480 μL of the 10 mM HEPES buffer in 0.15 M NaCl (pH = 7) and spin again at 14,000 × g for 10 min at RT. Perform this step 2x for a total of four spin-throughs.

5. Place filter device upside down into a new 2 mL snap-cap polypropylene tube, and centrifuge at 1000 × g for 2 min to collect LDL sample (final volume = about 30 μL).

6. Aliquot sample into appropriately labeled 1 mL tube and add 20 μL of water to each sample to achieve a final volume of 50 μL.

7. Preparation of 200 μM $CuCl_2$ stock solution in acetic acid (pH = 4)

    1. Prepare acetic acid solution (pH = 4): see step 1.1.9.1.

    2. Prepare a 144 mM $CuCl_2$ stock solution (see step 1.1.9.2), then aliquot 5.5 μL from the 144 mM $CuCl_2$ stock and dissolve in a final volume of 4 mL of acetic acid (pH = 4) to make the 200 μM solution.

8. Aliquot 2.7 μL from the 200 μM $CuCl_2$ stock solution and add it to six samples containing 50 μL of LDL to achieve a final $CuCl_2$ concentration of ~10 μM. Add 2.7 μL from an acetic acid solution (pH = 4) that does not contain $CuCl_2$ to six samples containing 50 μL of LDL to be used for the controls.

9. Incubate LDL samples for 2 h in an oven at 37 °C. After 2 h, bring the final volume to 100 μL for each sample with 10 mM HEPES buffer in 0.15 M NaCl (pH = 7). Immediately perform a TBARS assay.

10. Repeat steps 1.2.3–1.2.9 2x on two different days to test reproductivity of the TBARS assay.

3. Human serum

1. From a human serum sample, make aliquots of 94 μL each into 2 mL snap-cap polypropylene tubes and store samples at −80 °C.

2. Prepare a 35 mM $CuCl_2$ stock solution in acetic acid (pH = 4): see step 1.1.9.

3. Aliquot 6 μL from the $CuCl_2$ stock solution and add it to six samples containing 94 μL of human serum to make a final $CuCl_2$ concentration of about 2 mM. Add 6 μL of an acetic acid solution (pH = 4) that does not have $CuCl_2$ to six samples containing 94 μL of human serum to use as controls.

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4. Incubate human serum samples for 24 h in an oven at 37 °C and determine levels of oxidation with TBARS assay (section 4).

5. Repeat steps 1.3.2–1.3.4 2x on two separate days to determine reproducibility of the TBARS assay.

2. Reagent preparation

CAUTION: Thiobarbituric acid causes skin and eye irritation and maybe harmful by inhalation or skin absorption. Acetic acid can damage internal organs if inhaled. Prepare all acid solutions in a fume hood.

1. Preparation of 8.1% (w/v) sodium dodecylsulfate (SDS) solution

    1. Weight out 32.4 g of SDS and dissolve in 350 mL of DI water in a beaker. Use a magnetic stir bar to gently dissolve SDS and avoid making bubbles. Bring final volume to 400 mL with DI water and store SDS solution at RT.

       NOTE: Here, excess 8.1% SDS solution is prepared; however, for 96 samples, only about 20 mL of the 8.1% SDS solution are needed. Prepare this solution according to the number of samples being analyzed.

2. Preparation of 3.5 M sodium acetate buffer (pH = 4)

    1. Dilute 100 mL of glacial acetic acid in 350 mL of DI water in a beaker. Use a magnetic stir bar to gently dissolve it.

    2. Prepare a 6.5 M NaOH solution using sodium hydroxide beads in water. Dissolve 13 g of NaOH beads in 40 mL of DI water and bring to a final volume of 50 mL with DI water.

    3. Slowly add about 46 mL of the 6.5 M NaOH solution to the acetic acid solution while mixing with the stir bar (this should raise the pH to 4, but confirm by slowly adding the NaOH solution while measuring using a pH meter).

    4. Bring final volume to 500 mL with DI water and store sodium acetate buffer at RT.

3. Preparation of 0.8% aqueous solution of thiobarbituric acid (adjusted to pH = 4)

   NOTE: In this step, preparation of thiobarbituric acid is optimized for large volumes, since a large number of samples is going to be analyzed (108 samples, not including the standards). Prepare this solution depending on the number of samples planned for analysis.

    1. Prepare a 5 M sodium hydroxide solution using sodium hydroxide beads and water: dissolve 4 g of sodium hydroxide beads in a final volume of 20 mL of water. Store in a plastic container. This solution should be freshly prepared for each batch.

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2. Weight 4 g of thiobarbituric acid and add 450 mL of DI water. Use a magnetic stir bar to gently dissolve it.

   NOTE: This solution will eventually be brought to a 500 mL total volume.

3. While dissolving thiobarbituric acid with a stir bar, add (slowly and in a dropwise manner) about 3 mL of the 5 M NaOH solution in 100 µL increments. After adding the NaOH solution, the thiobarbituric acid particles will start to dissolve.

4. If the thiobarbituric acid particles still have not fully dissolved, add more of the 5 M NaOH solution in 100 µL increments until all thiobarbituric acid particles are fully dissolved. For this particular volume of solution, a total of 4 mL of the 5 M NaOH solution is added to fully dissolve the thiobarbituric acid particles.

   NOTE: At this concentration, thiobarbituric acid will not fully dissolve unless the pH is nearly 4.

5. Stop adding NaOH after all the thiobarbituric acid has fully dissolved. Avoid exceeding a pH of 4. The final pH can be verified by taking 1 µL from the mixing thiobarbituric acid solution and placing it onto pH paper.

6. Bring final volume to 500 mL with DI water and store aqueous 0.8% thiobarbituric acid solution at RT.

3. Malondialdehyde bis(dimethyl acetal) standard sample preparation

NOTE: Malondialdehyde (MDA) is unstable and not commercially available. However, there are different chemical forms of MDA that are commercially available, such as MDA tetrabutylammonium salt, MDA bis(dimethyl acetal), and MDA bis(diethyl acetal). Of these three chemical forms, MDA bis(dimethyl acetal) is used here, because a majority of studies use this same standard[21,22]. If choosing to use the other two chemical forms of MDA, prior validation of their suitability should be carried out.

1. Prepare a 550 µM MDA bis(dimethyl acetal) stock solution by diluting 92 µL of pure MDA bis(dimethyl acetal) in 1 L of DI water. Use a magnetic stir bar to mix the solution thoroughly for 10 min. Store solution at 4 °C and use within 1 month.

2. Prepare a 200 µM MDA bis(dimethyl acetal) by diluting 726 µL from the 550 µM MDA bis(dimethyl acetal) stock in 1274 µL of DI water. This 200 µM MDA bis(dimethyl acetal) solution should be prepared fresh every time a TBARS assay is performed.

3. Standard curve preparation: take eight 2 mL snap-cap polypropylene tubes and label them with letters A through H. Add MDA bis(dimethyl acetal) from the 200 µM stock and dilute in water as described in Table 1.

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4. Take eight glass tubes (13 mm × 100 mm) and label them A–H, then add 100 μL of standard to the corresponding tubes. Perform six replicates for the blank standard (sample A) to calculate the limits of detection of the TBARS assay.

   NOTE: The protocol can be paused here for no more than 1 h.

4. TBARS assay

NOTE: Once the TBARS assay is started, it should be finished without stopping.

1. Take as many glass tubes as needed for the number of samples to be analyzed and label them with the names of the samples. Then, add 100 μL of each prepared sample (as described above) to each glass tube.
2. Add 200 μL of 8.1% SDS to each sample and standard and gently swirl the glass tube in a circular motion to mix the sample.
3. Add 1.5 mL of the 3.5 M sodium acetate buffer (pH = 4) to each sample and standard.
4. Add 1.5 mL of the aqueous 0.8% thiobarbituric acid solution (pH = 4) to each sample and standard.
5. Bring the final volume to 4 mL for each sample and standard by adding 700 μL of DI water.
6. Tightly cap each glass tube and incubate in a heating block set to 95 °C for 1 h. Cover the glass tubes with aluminum foil to prevent condensation at the tops of the tubes.
7. Remove the glass tubes from the heating block and incubate on ice for 30 min.
8. Centrifuge samples and standards at 1500 × g for 10 min at 4 °C. After centrifugation, keep the glass tubes containing the samples and standards at RT.

   NOTE: Keeping the samples on ice or at 4 °C will cause the entire sample or standard to precipitate.

9. Immediately after centrifugation, aliquot 150 μL of supernatant from each tube and place into a separate well of a 96 well plate.
10. Remove any bubbles from each well using a pipette tip.

    NOTE: The presence of bubbles will yield inconsistent absorbance readings, leading to high assay imprecision.

11. Read absorbances at 532 nm. Subtract the average absorbance reading of the blank samples from all other absorbance readings.
12. Create a standard curve by plotting the blank-subtracted absorbance readings at 532 nm vs. the known concentration of each standard. Fit the data points using linear regression. Calculate unknown sample concentrations by using the equation of the linear regression line obtained from the standard curve.

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Representative Results

Under acidic conditions (pH = 4) and at 95 °C, malondialdehyde (MDA) bis(dimethyl acetal) yields MDA[23]. MDA and closely related chemical congeners react with two molecules of thiobarbituric acid (TBA) to produce compounds called thiobarbituric acid reactive substances (TBARS), which give a red-pink color and have an absorbance λ_{max} at 532 nm (Figure 1, Figure 2). Using MDA bis (dimethyl acetal) as the standard, standard curves were generated (Figure 3, Table 1) to determine the limits of detection and sensitivity of the assay and levels of oxidation in three different biological samples. A total of nine TBARS assays were performed to determine the levels of oxidation in the three different samples on different days. Hence, a total of nine standard curves were generated, as shown in Figure 3. The least squares procedure[24] was used to determine the standard deviations of the slope and the y-intercept, which were 8.67×10^{-6} and 5.66×10^{-4}, respectively.

The limits of detection of the TBARS assay were determined according to standard analytical procedures[25] by measuring absorbances of the blank samples (six experimental replicates with two technical replicates per experimental replicate) on three different days. The minimum distinguishable analytical signal (S_m) was determined by summing the mean of the blank signal $(\bar{S}_{bl})$ plus a multiple k of the standard deviation of the blank (ks_{bl}), where $k = 3$. That is, $S_m = \bar{S}_{bl} + ks_{bl}$. Using S_m and the slope of the standard curve (m), the detection limit (c_m) was calculated as $c_m = (S_m - \bar{S}_{bl})/m$. The resulting data of the blank samples on three different days shows that the minimum concentration of TBARS substance needed to give a detectable non-noise absorbance signal is 1.1 μM (Table 2). The sensitivity of the TBARS assay is about 0.00160 absorbance units/μM, which is the ability of the assay to distinguish differences in analyte concentration (Table 2).

To illustrate the applicability of the TBARS assay in detecting changes in lipid peroxidation in various biological matrices, $CuCl_2$ was used to induce the in vitro oxidation of human serum, HepG2 cell lysates, and low density lipoproteins. These biological samples used here are prototypes of biological matrices. For example, based on the results presented here for HepG2 cell lysates, it is reasonable to expect that this assay will work with other types of cell lysate; however, it would need to be analytically validated for this purpose. Also, of the three biological matrices used here, it is common for certain types of samples to exhibit low endogenous concentrations of TBARS. For example, TBARS for HepG2 cell lysates that were not treated with $CuCl_2$ were just above the limit of detection of the assay (about 2 μM; Figure 4). As would be expected in the presence of low signal-to-noise ratios, the standard deviation and coefficient of variation for this particular sample is relatively high (Table 3). However, as the signal increases as a result of Cu(II) mediated oxidation, the coefficient of variation becomes lower. In general, as the absorbance increases beyond the detection limit, assay reproducibility improves (Table 3).

For the purposes of this protocol, there was no desire to use antioxidants to mask the in vitro Cu(II)-mediated oxidation of biological samples. Commercially prepared low density lipoprotein (LDL) may contain 0.01% EDTA. EDTA will prevent Cu(II)-mediated oxidation of LDL but not necessarily other metal-mediated oxidation reactions[26,27]. A TBARS assay was performed on LDL samples containing EDTA, and the levels of TBARS did not change

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between the Cu(II)-treated and untreated LDL samples (Figure 5A). However, after EDTA was removed by spin filtration (see step 1.2.3–1.2.5), LDL underwent Cu(II)-mediated oxidation, as detected by the TBARS assay (Figure 5B).

The normal range of lipid peroxidation products in the human serum from healthy donors expressed in terms of MDA is between 1.80–3.94 μM[28]. To illustrate the dynamic range of the TBARS assay in human serum, a concentration of 2 mM Cu(II) ions was added to the samples, followed by incubation for 24 h at 37 °C. This resulted in a 6x–7x increase in TBARS (Figure 6).

Discussion

Despite its limitations[1,3,4,7,8,9,10,12,13,14,15,19] and a lack of suitability for comparison between laboratories, the TBARS assay is one of the oldest[29,30] but most widely used assays to measure oxidative stress in biological samples. The TBARS assay is a straightforward method that only takes about 2 h to perform, once all the required reagents have been prepared. Here, we have described in detail how this assay, including standard curve, can be performed many times in an economical way (about $3.50 USD for 96 samples), without having to buy an expensive kit for every batch of samples.

All steps of the assay are critical, but there are some steps that require extra attention. For instance, the pH of the thiobarbituric acid should not be higher than 4. Precautions should be taken when adding the sodium hydroxide solution to the thiobarbituric acid and avoid obtaining a pH of greater than 4. An acidic environment is required for the reaction between MDA and TBA to occur, and the MDA standard is released from MDA bis (dimethyl acetal) by acid-catalyzed hydrolysis. Hence, a high pH may lead to unpredictable and highly variable results[31].

Also, while this may be obvious to some readers, it is also critical to remove any bubbles in the 96 well plate before measuring the absorbance. The presence of bubbles will yield high absorbance values and differences between replicates, leading to high percentage of CVs. Additionally, after the 1 h incubation at 95 °C, samples should not be incubated longer than 30 min on ice, since this will precipitate the entire sample, and collecting a precipitate-free supernatant will be difficult to accomplish. Notably, there are no good stopping points once the TBARS assay has been started. It should be completed once initiated. Finally, there are many possible methodological variations that can be applied to this assay. The general protocol described here can be further adapted (and validated) for specific applications, including those in which the addition of radical scavengers or other types of antioxidants prior to analysis is required.

While the TBARS assay is popular, it is important to realize that it is not a molecularly specific assay. Numerous chemically reactive carbonyl-containing organic molecules, including those derived from oxidized biomolecules other than lipids, can react with TBA and are thus counted as TBARS[1,32,33,34]. In addition, the limits of detection of the absorbance-based TBARS assay do not get much better than about 1.1 μM, as determined by this method. However, the limits of detection can be improved by using other detection

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methods. For instance, spectrofluorometry with excitation at 520 nm and emission at 550 nm offer higher sensitivity and better limits of detection, as previously suggested by Jo and Ahn[35]. Mass spectrometry-based methods can dramatically improve both specificity and limits of detection. For example, a GC-MS/MS with electron-capture negative-ion chemical ionization (ECNICI) method has been used to detect the pentafluorobenzyl derivative of MDA in human serum and urine samples, with limits of detection of 2×10^{-18} mol MDA on column[36]. Here, the chromatographic separation, in combination with tandem mass spectrometry, dramatically improves the molecular specificity of the assay, as well.

Nevertheless, as with other measurements of oxidative processes within biological samples[37,38], preanalytical sample handling is critical to the outcome of TBARS measurements. For example, blood plasma storage at −20 °C results in slow but dramatic increases in MDA concentrations[39,40]. Thus, exposure of biological samples to thawed or even partially thawed conditions for anything but a minimal amount of time should be assumed to cause artifactual elevation of TBARS levels. This means that even modest variability in the preanalytical handling and storage of biospecimens that are to be compared using the TBARS assay must be avoided.

Given these caveats related to preanalytical variability as well as limited sensitivity and specificity, it is recommended that the absorbance-based TBARS assay only be used for intra-laboratory general assessment or range-finding experiments. These applications include studies in which relative TBARS levels are directly compared between one or more groups of biologically similar samples that are processed or stored together and separated by only a single variable that is fully controlled by researchers.

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References

1. Tsikas D Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Analytical Biochemistry. 524, 13–30 (2017). [PubMed: 27789233]
2. Ohkawa H, Ohishi N, Yagi K. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. Journal of Lipid Research. 19 (8), 1053–1057 (1978). [PubMed: 103988]
3. Khoubnasabjafari M, Soleymani J, Jouyban A Avoid Using Spectrophotometric Determination of Malondialdehyde as a Biomarker of Oxidative Stress. Biomarkers in Medicine. 12 (6), 551–554 (2018). [PubMed: 29873547]
4. Morales M, Munné-Bosch S Malondialdehyde: Facts and artifacts. Plant Physiology. 180 (3), 1246–1250 (2019). [PubMed: 31253746]
5. Devasagayam TPA, Boloor KK, Ramasarma T Methods for estimating lipid peroxidation: An analysis of merits and demerits. Indian Journal of Biochemistry and Biophysics. 40 (5), 300–308 (2003). [PubMed: 22900323]
6. Dasgupta A, Klein K Methods for Measuring Oxidative Stress in the Laboratory. In Antioxidants in Food, Vitamins and Supplements. 19–40 (2014).

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7. Wade CR, van Rij AM Plasma malondialdehyde, lipid peroxides, and the thiobarbituric acid reaction. Clinical Chemistry. 35 (2), 336–336 (1989).
8. Khoubnasabjafari M, Ansarin K, Jouyban A Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders. BioImpacts. 5 (3), 123–127 (2015). [PubMed: 26457249]
9. Khoubnasabjafari M, Ansarin K, Jouyban A Comments Concerning "Comparison of Airway and Systemic Malondialdehyde Levels for Assessment of Oxidative Stress in Cystic Fibrosis". Lung. 193 (5), 867–868 (2015). [PubMed: 26238860]
10. Khoubnasabjafari M, Ansarin K, Vaez-Gharamaleki J, Jouyban A Comments on "Salivary 8-hydroxy-2-deoxyguanosine, malondialdehyde, vitamin C, and vitamin E in oral pre-cancer and cancer: diagnostic value and free radical mechanism of action". Clinical Oral Investigations. 20 (2), 395–396 (2016). [PubMed: 26662573]
11. Khoubnasabjafari M, Ansarin K, Jouyban A Comments on "An Investigation into the Serum Thioredoxin, Superoxide Dismutase, Malondialdehyde, and Advanced Oxidation Protein Products in Patients with Breast Cancer". Annals of Surgical Oncology. 24, S573–S576 (2017).
12. Azizi S et al. Effects of analytical procedures on the repeatability of malondialdehyde determinations in biological samples. Pharmaceutical Sciences. 23 (3), 193–197 (2017).
13. Azizi S et al. A possible reason for the low reproducibility of malondialdehyde determinations in biological samples. Bioanalysis. 8 (21), 2179–2181 (2016). [PubMed: 27667475]
14. Wasowicz W, Neve J, Peretz A Optimized steps in fluorometric determination of thiobarbituric acid- reactive substances in serum: Importance of extraction pH and influence of sample preservation and storage. Clinical Chemistry. 39 (12), 2522–2526 (1993). [PubMed: 8252725]
15. Jentzsch AM, Bachmann H, Fürst P, Biesalski HK Improved analysis of malondialdehyde in human body fluids. Free Radical Biology and Medicine. 20 (2), 251–256 (1996). [PubMed: 8746446]
16. Buege JA, Aust SD Microsomal lipid peroxidation. Methods in Enzymology. 52, 302–310 (1978). [PubMed: 672633]
17. Gutteridge JMC Free-Radical Damage to Lipids, Amino-Acids, Carbohydrates and Nucleic-Acids Determined by Thiobarbituric Acid Reactivity. International Journal of Biochemistry. 14 (7), 649–653 (1982). [PubMed: 7106362]
18. Khoubnasabjafari M, Ansarin K, Jouyban A Salivary malondialdehyde as an oxidative stress biomarker in oral and systemic diseases. J Dent Res Dent Clin Dent Prospects. 10 (2), 71–74 (2016). [PubMed: 27429721]
19. Halliwell B, Whiteman M Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? British Journal of Pharmacology. 142 (2), 231–255 (2004). [PubMed: 15155533]
20. Lee R et al. "Evaluating oxidative stress in human cardiovascular disease: methodological aspects and considerations." Current medicinal chemistry. 19 (16), 2504–2520 (2012). [PubMed: 22489713]
21. Morel DW, Hessler JR, Chisolm GM Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. Journal of Lipid Research. 24 (8), 1070–1076 (1983). [PubMed: 6415194]
22. Guzmán-Chozas M, Vicario-Romero IM, Guillén-Sans R 2-thiobarbituric acid test for lipid oxidation in food: Synthesis and spectroscopic study of 2-thiobarbituric acid-malonaldehyde adduct. Journal of the American Oil Chemists Society. 75 (12), 1711–1715 (1998).
23. Shibata T, et al. Identification of a lipid peroxidation product as a potential trigger of the p53 pathway. Journal of Biological Chemistry. 281(2), 1196–1204 (2006). [PubMed: 16251187]
24. Skoog DA, West DM, Holler FJ, and Crouch SR Sampling, standardization, and calibration. In Fundamentals of Analytical Chemistry. 9th ed., Ch. 7, 153–196, Cengage - Brooks/Cole, Belmont, CA, (2014).
25. Skoog DA, Holler FJ, and Crouch SR Introduction. In Principles of Instrumental Analysis. 6th ed., Ch. 1, 1–24, Cengage - Brooks/Cole, Belmont, CA, (2007).
26. Seibig S,, Van Eldik R Kinetics of [FeII(edta)] Oxidation by Molecular Oxygen Revisited. New Evidence for a Multistep Mechanism. Inorganic Chemistry. 36 (18), 4115–4120 (1997).

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27. Jeffs JW et al. Delta-S-Cys-Albumin: A Lab Test that Quantifies Cumulative Exposure of Archived Human Blood Plasma and Serum Samples to Thawed Conditions. Molecular & Cellular Proteomics. 18 (10), 2121–2137 (2019). [PubMed: 31324658]
28. Yagi K Simple Assay for the Level of Total Lipid Peroxides in Serum or Plasma. In Free Radical and Antioxidant Protocols. Methods in Molecular Biology. Edited by Armstrong D, 101–106, Humana Press. Totowa, NJ (1998).
29. Bernheim F, Bernheim MLC, Wilbur KM The reaction between thiobarbituric acid and the oxidation products of certain lipides. Journal of Biological Chemistry. 174 (1), 257–264 (1948). [PubMed: 18914082]
30. Wilbur KM, Bernheim F, Shapiro OW The thiobarbituric acid reagent as a test for the oxidation of unsaturated fatty acids by various agents. Archives of Biochemistry. 24 (2), 305–313 (1949). [PubMed: 15405719]
31. Kwon TW, Watts BM Determination of malonaldehyde by ultraviolet spectrophotometry. Journal of Food Science. 28 (6), 627–630 (1963).
32. Esterbauer H, Schaur FJ, Zollner H Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radical Biology & Medicine. 11 (1), 81–128 (1991). [PubMed: 1937131]
33. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A Biomarkers of oxidative damage in human disease. Clinical Chemistry. 52 (4), 601–623 (2006). [PubMed: 16484333]
34. Jentzsch AM, Bachmann H, Fürst P, Biesalski HK Improved analysis of malondialdehyde in human body fluids. Free Radical Biology and Medicine. 20 (2), 251–256 (1996). [PubMed: 8746446]
35. Jo C, Ahn DU Fluorometric Analysis of 2-Thiobarbituric Acid Reactive Substances in Turkey. Poultry Science. 77 (3), 475–480 (1998).
36. Tsikas D et al. Development, validation and biomedical applications of stable-isotope dilution GC-MS and GC-MS/MS techniques for circulating malondialdehyde (MDA) after pentafluorobenzyl bromide derivatization: MDA as a biomarker of oxidative stress and its relation to 15(S)-8-iso-prostaglandin F2α and nitric oxide (NO). Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences. 1019, 95–111 (2016). [PubMed: 26522895]
37. Barden AE, Mas E, Croft KD, Phillips M, Mori TA Minimizing artifactual elevation of lipid peroxidation products (F 2-isoprostanes) in plasma during collection and storage. Analytical Biochemistry. 449 (1), 129–131 (2014). [PubMed: 24380789]
38. Jeffs JW, Ferdosi S, Yassine HN, Borges CR Ex vivo instability of glycated albumin: A role for autoxidative glycation. Archives of Biochemistry and Biophysics. 629, 36–42 (2017). [PubMed: 28712850]
39. Lee DM Malondialdehyde in Stored Plasma. Biochemical and Biophysical Research Communications. 95 (4), 1663–1672 (1980). [PubMed: 6774730]
40. Tsikas D et al. Simultaneous GC-MS/MS measurement of malondialdehyde and 4-hydroxy-2-nonenal in human plasma: Effects of long-term L-arginine administration. Analytical Biochemistry. 524, 31–44 (2017). [PubMed: 27530652]

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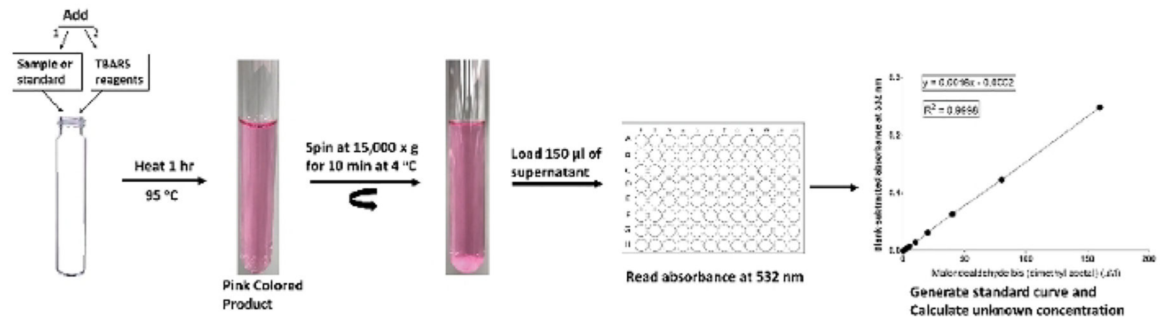

Figure 1: Thiobarbituric acid reactive substances assay schematic.

One hundred microliters of sample or standard are added to a 13 mm × 100 mm glass tube, followed by addition of thiobarbituric acid reactive substances (TBARS) reagents. After incubation at 95 °C for 1 h, samples and standards are incubated in ice for 30 min, then centrifuged at 1,500 × g for 10 min at 4 °C. One hundred fifty microliters of sample or standard supernatant are loaded onto a 96 well plate, and absorbance is measured at 532 nm. Unknown sample concentration is calculated using the equation of the standard curve.

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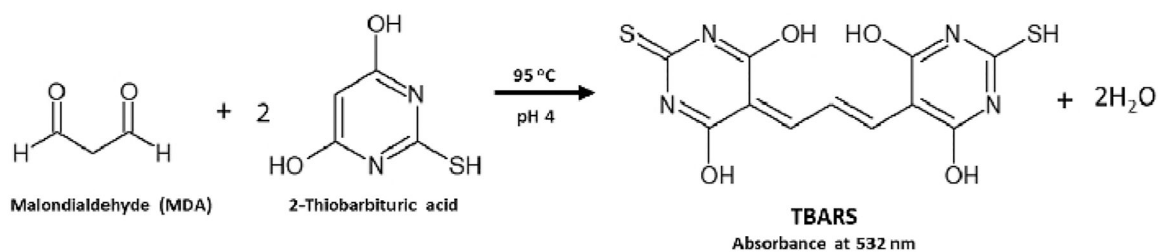

Figure 2: Archetype thiobarbituric acid reactive substances reaction.

Malondialdehyde bis(dimethyl acetal) yields malondialdehyde under acid-catalyzed hydrolysis[1]. Released Malondialdehyde (MDA) then reacts with two molecules of 2-thiobarbituric acid (TBA) (pH = 4 and 95 °C) to form MDA-TBA_2 adducts that give a red-pink color and can be measured spectrophotometrically at 532 nm. Because other molecules besides MDA that are derived from oxidized lipids can also react with TBA, the absorbance measurement at 532 nm is simply referred to as a measurement of thiobarbituric acid reactive substances, or TBARS.

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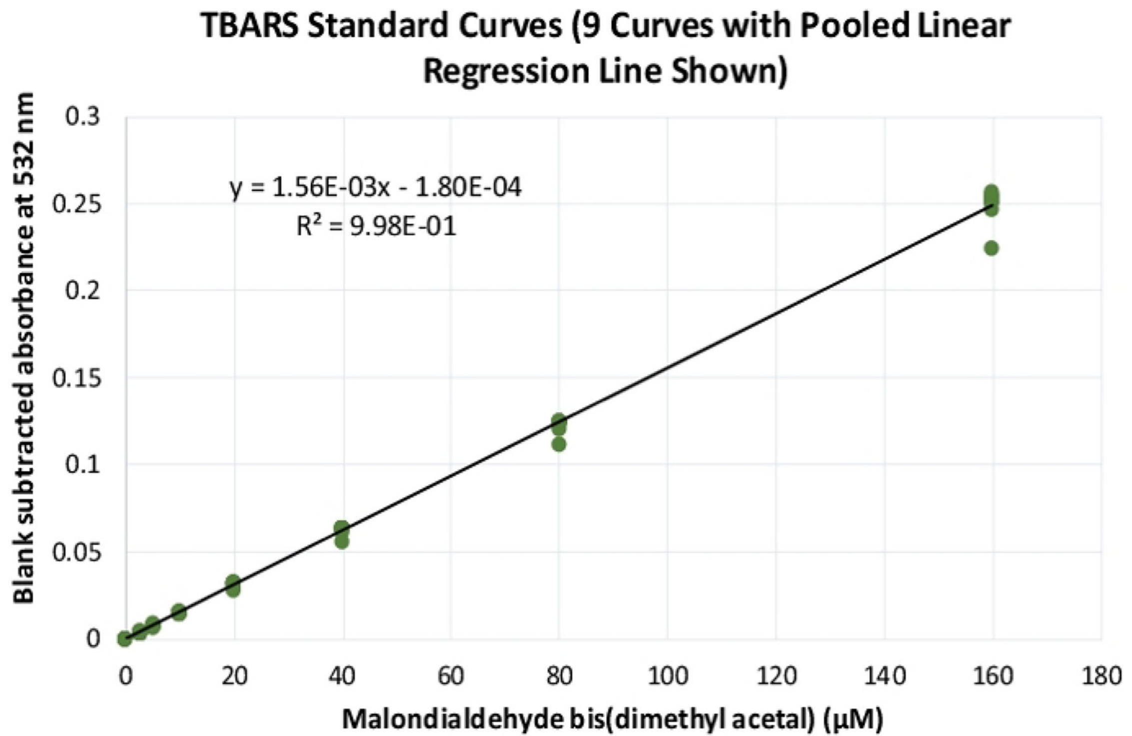

Figure 3: Malondialdehyde bis(dimethyl acetal) colorimetric standard curves.

Figure shows nine standard curves as created on different days. Some points overlap and cannot be distinguished from one another. Malondialdehyde bis(dimethyl acetal) was fortified into calibrator samples at 0, 2.5, 5, 10, 20, 40, 80, and 160 µM (as shown in Table 1; n = 1 per concentration point per day). Absorbance was measured at 532 nm, with the average absorbance of the blank samples subtracted from all measurements in that batch, including unknowns. Each day, the equation generated by least squares linear regression was used to determine TBARS in biological samples. For all nine standard curves combined, the standard deviation of the slope was 8.67×10^{-6}, and the standard deviation of the y-intercept was 5.66×10^{-4}. Standard deviations of the slope and y-intercept were calculated using the least squares procedure[24].

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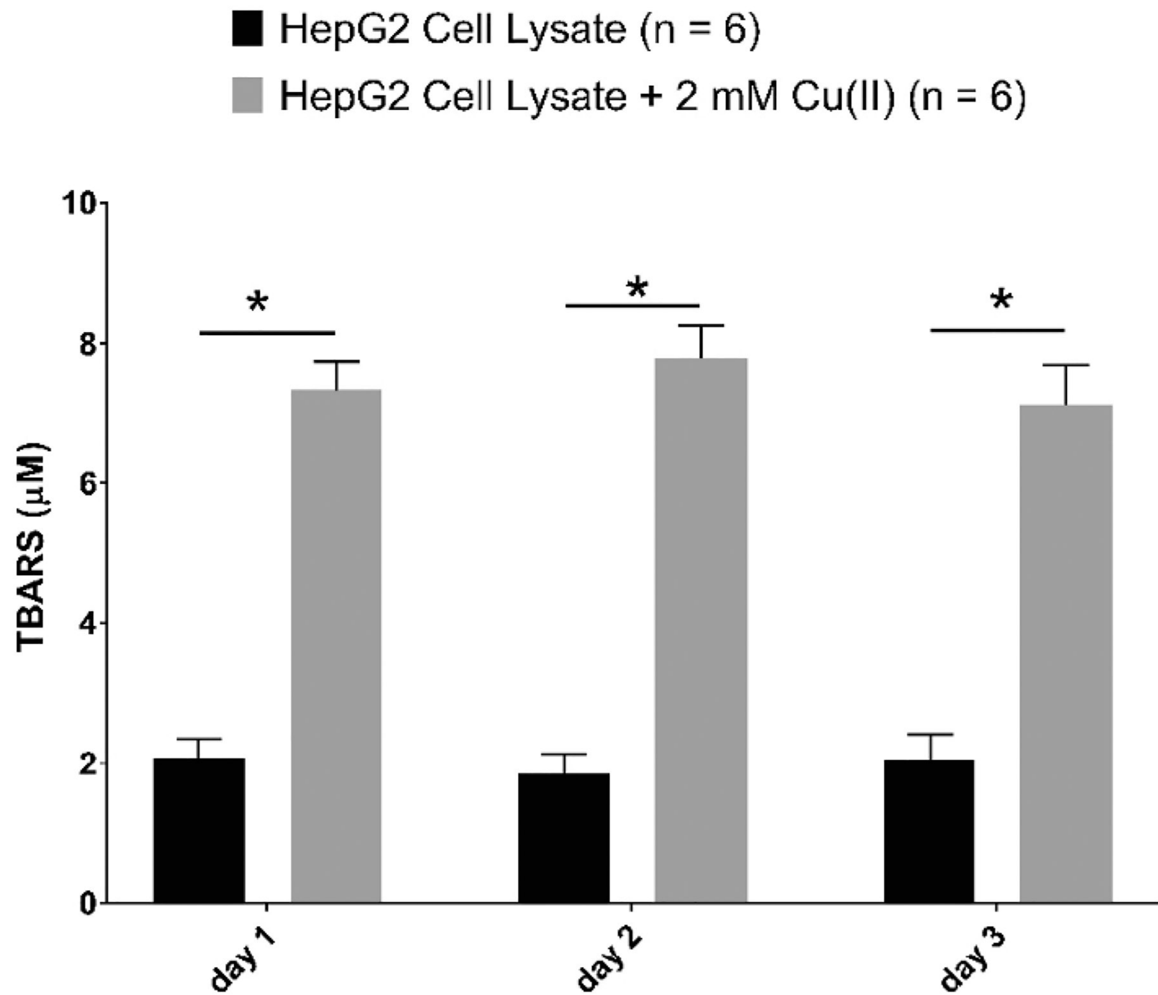

Figure 4: Oxidation in HepG2 lysates detected by TBARS.

Six HepG2 cell lysate samples were incubated with 2 mM $CuCl_2$ [HepG2 cell lysate + 2 mM Cu(II)] and six samples were incubated in a solution without $CuCl_2$ (HepG2 cell lysate) for 24 h at 37 °C. After incubation, the TBARS assay was performed on the 12 samples. This procedure was repeated 2x for a total of three different days. Error bars represent SD. Asterisk indicates statistically significant differences between control and Cu(II)-treated lysates ($p < 0.001$). Statistical significance was determined using a Mann Whitney U test in GraphPad.

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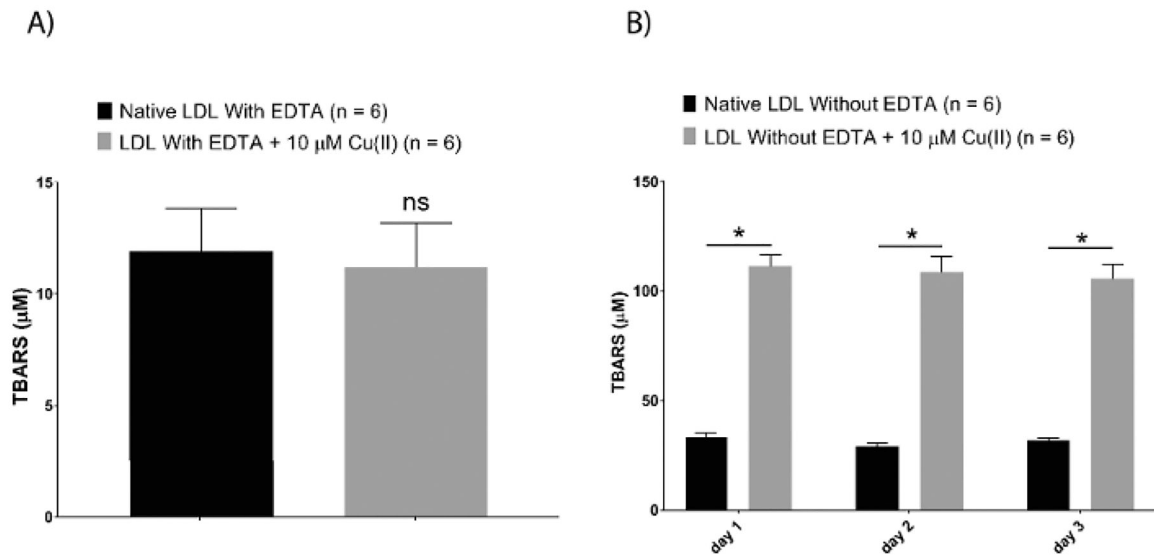

Figure 5: Oxidation in low density lipoprotein detected by TBARS.

(**A**) TBARS assay conducted in LDL samples containing 0.01% EDTA. Six LDL samples were incubated with 10 µM $CuCl_2$ [LDL + 10 µM Cu(II)], and six samples were incubated with a control solution with no $CuCl_2$ added (Native LDL) for 2 h at 37 °C. Then, a TBARS assay was performed on the 12 samples. "ns" represents no statistical significance. (**B**) LDL was spin filtered using a centrifugal spin filter device to remove EDTA. Then, incubation with and without added Cu(II) was performed again as described for (A). The TBARS assay was performed immediately afterward. This same procedure was repeated 2x for a total of 3 days. Error bars represent SD. Asterisk indicates statistically significant differences between control and Cu(II)-treated LDL samples ($p < 0.001$). Statistical significance was determined using the Mann Whitney U test in GraphPad.

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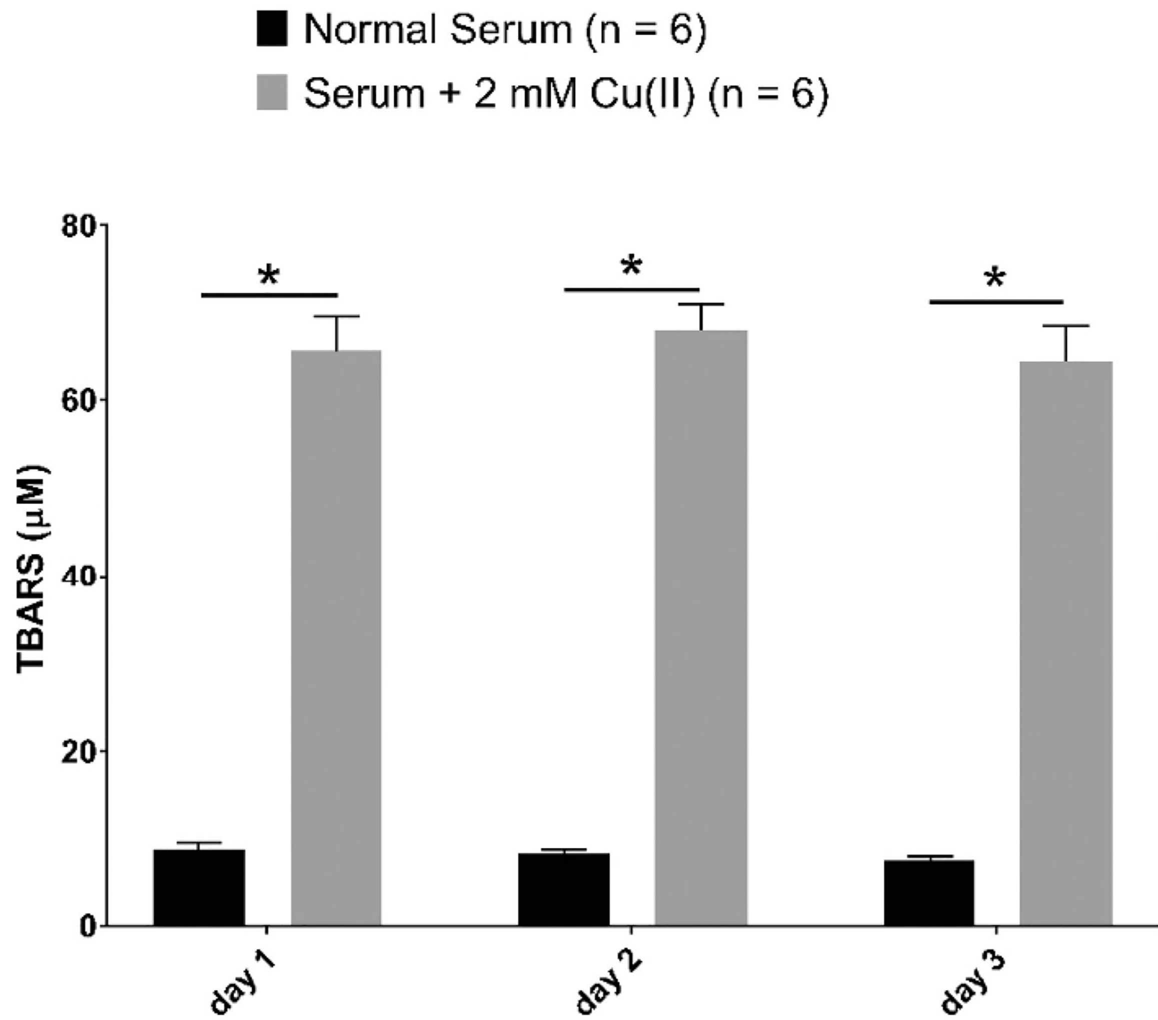

Figure 6: Lipid peroxidation in human serum samples detected by TBARS.

Six human serum samples were incubated with 2 mM $CuCl_2$ [serum + 2 mM Cu(II)], and six samples were incubated with a solution that did not have any added $CuCl_2$ (normal serum) for 24 h at 37 °C. After incubation, the TBARS assay was performed on the 12 samples. This procedure was repeated on two additional days. Error bars represent SD. Asterisk indicates statistically significant differences between control and Cu(II)-treated serum samples ($p < 0.001$). Statistical significance was determined using the Mann Whitney U test in GraphPad.

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Table 1:

Malondialdehyde bis(dimethyl acetal) standard sample preparation.

From the freshly prepared 200 μM malondialdehyde bis(dimethyl acetal), aliquot the suggested volumes to reach the final concentration for the standard curve. It is recommended to perform at least six replicates of the blank sample (A) per day to determine the limits of detection of the method.

Glass Tube	200 μM MDA bis (dimethyl acetal) (μL)	Water (μL)	MDA bis (dimethyl acetal) Final Concentration (μM)
A[a]	0	1000	0
B	12.5	987.5	2.5
C	25	975	5
D	50	950	10
E	100	900	20
F	200	800	40
G	400	600	80
H	800	200	160

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Table 2:

Detection limits of the TBARS assay.

Day	Absorbance[a]	s_{bl}[b]	S_m[c]	Sensitivity (absorbance units/μM)[d]	c_m (μM)[e]
1 (n = 6)	0.0412	0.000612	0.0430	0.00160	1.14
2 (n = 6)	0.0415	0.000632	0.0433	0.00160	1.18
3 (n = 6)	0.0413	0.000605	0.0431	0.00160	1.13
All three days (n = 18)	0.0413	0.000589	0.0431	0.00160	1.10

[a] Absorbance of the blank samples on three different days with 6 replicates per day.

[b] s_{bl} = Standard deviation of the absorbance of the blank samples.

[c] S_m = Minimum distinguishable analytical signal, which was determined by summing the mean of the blank signal $(\bar{S}_{bl})$ plus a multiple k of the standard deviation of the blank (ks_{bl}), where k = 3. That is; $S_m = \bar{S}_{bl} + ks_{bl}$.

[d] Sensitivity of the TBARS assay, which is the slope of the standard curve.

[e] c_m = Limits of detection, which was calculated as $c_m = (S_m - \bar{S}_{bl})/m$, where m = the slope of the standard curve.

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Table 3:

Analytical reproducibility of TBARS in three different biological samples.

Low density lipoprotein		**Human Serum**		**HepG2 Cell Lysate**	
Day	**% CV**	**Day**	**% CV**	**Day**	**% CV**
1 (n = 6)	5.6	1 (n = 6)	7.9	1 (n = 6)	12.6
2 (n = 6)	5.4	2 (n = 6)	7.2	2 (n = 6)	15.8
3 (n = 6)	3.9	3 (n = 6)	7.0	3 (n = 6)	17.7
All three days (n = 18)[a]	7.4	All three days (n = 18)	9.8	All three days (n = 18)	15.5[b]
With 10 µM CuCl2		**With 2 mM CuCl $_2$**		**With 2 mM CuCl $_2$**	
1 (n = 6)	4.5	1 (n = 6)	6.0	1 (n = 6)	5.8
2 (n = 6)	6.5	2 (n = 6)	4.3	2 (n = 6)	6.0
3 (n = 6)	6.7	3 (n = 6)	6.2	3 (n = 6)	8.0
All three days (n = 18)	6.1	All three days (n = 18)	5.6	All three days (n = 18)	7.3

[a]Interday precision was calculated by pooling data from all three days.

[b]Precision was limited due to results being near the assay LOD.