Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61122R1
Full Title:	Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituic Acid Reactive Substances Assay
Section/Category:	JoVE Chemistry
Keywords:	Thiobarbituric Acid Reactive Substances (TBARS); Oxidation; Lipid Peroxidation; Malondialdehyde (MDA); Thiobarbituric Acid (TBA).
Corresponding Author:	Chad Borges Arizona State University Tempe, AZ UNITED STATES
Corresponding Author's Institution:	Arizona State University
Corresponding Author E-Mail:	Chad.Borges@asu.edu
Order of Authors:	Jesús Salvador Aguilar Díaz de león
	Chad Borges
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tempe, Arizona, United States

Cover Letter

Chad R. Borges, Ph.D.
Assistant Professor
School of Molecular Sciences
The Biodesign Institute at Arizona State University -
Center for Personalized Diagnostics
P.O. Box 876401
Tempe, AZ 85287
Email: chad.borges@asu.edu
Office Phone: 480-727-9928

13 December, 2019

Dear *Journal of Visualized Experiments (JOVE)* Editorial Board:

We hereby respectfully submit a manuscript entitled, "*Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay*" for your consideration as a publication in the *Journal of Visualized Experiments (JOVE)*.

The Thiobarbituric Acid Reactive Substances (TBARS) assay is used to measure lipid peroxidation in biological fluids and is often considered a good indicator of the levels of oxidative stress in a biological sample. Unfortunately, this assay can only be found as an expensive kit (over $400 dollars). Moreover, the instructions with such kits do not provide detailed information on most of the concentrations of the reagents used. To make matters worse, the reagents provided can only be used for one experiment because only one colorimetric standard curve can be made per kit. 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 assay. Some researchers in the past have vaguely described how to perform the TBARS assay, 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. We have worked out how to perform the TBARS assay without an expensive kit using individually purchased chemicals. Our TBARS assay can be performed multiple times in an economical way. We have used our TBARS assay to determine levels of lipid peroxidation in human serum, cell lysates, and low-density lipoproteins, and have found it to work easily and reproducibly.

We feel that the *Journal of Visualized Experiments* represents the ideal publication forum from which to submit a detail protocol on this kit-free TBARS assay.

Thank you for your consideration.

Sincerely,

Chad Borges

Chad Borges, Ph.D.

TITLE:

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

AUTHORS AND AFFILIATIONS:

Jesus Aguilar Diaz De Leon, Chad R. Borges

School of Molecular Sciences, The Biodesign Institute - Center for Personalized Diagnostics, Arizona State University, Tempe, AZ, USA

Corresponding Author:

Chad R. Borges (chad.borges@asu.edu)

Email Address of Co-author:

Jesus Aguilar Diaz De Leon (jersus.aguilar@asu.edu)

KEYWORDS:

thiobarbituric acid reactive substances, TBARS, oxidation, lipid peroxidation, malondialdehyde, MDA, thiobarbituric acid, TBA, oxidative stress

SUMMARY:

The goal of the thiobarbituric acid reactive substances assay is to assess oxidative stress in biological samples by measuring the production of lipid peroxidation products, primarily malondialdehyde, using visible wavelength spectrophotometry at 532 nm. The method described here can be applied to human serum, cell lysates, and low density lipoproteins.

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.

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. 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[3,4,7-11]. 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 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

1.1. **HepG2 cell lysates**

1.1.1. Seed about 10 x 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.

1.1.2. 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.

1.1.3. 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 to 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.

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

1.1.5. 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.

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

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

1.1.8. Concentrate cell lysate supernatant four-fold using a Speed Vac at 50 ^{0}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 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 1.1.5 and 1.1.6 can then be performed.

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

1.1.9.1. 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.

1.1.9.2. 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.

1.1.10. 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.

1.1.11. 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. Repeat steps 1.1.8 and 1.1.9 2x on separate days to check the reproducibility of the TBARS assay for HepG2 cell lysates.

1.2. **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.

1.2.1. 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.

1.2.2. 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.

1.2.3. 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.

1.2.4. Spin samples at 14,000 x 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 x g for 10 min at RT. Perform this step 2x for a total of four spin-throughs.

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

1.2.6. 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.

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

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

1.2.7.2. Prepare a 144 mM $CuCl_2$ stock solution (see step 1.1.8.2), 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.

1.2.8. 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.

1.2.9. 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. Repeat steps 1.2.3–1.2.8 2x on two different days to test reproductivity of the TBARS assay.

1.3. **Human serum**

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

1.3.2. Prepare a 35 mM $CuCl_2$ stock solution in acetic acid (pH = 4): see step 1.1.8.

1.3.3. 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.

1.3.4. Incubate human serum samples for 24 h in an oven at 37 °C and determine levels of oxidation with TBARS assay (section 4). Repeat steps 1.3.2–1.3.3 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.

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

2.1.1. 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.

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

2.2.1. 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.

2.2.2. 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.

2.2.3. 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).

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

2.3. 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 analyzed (108 samples, not including the standards). Prepare this solution depending on the number of samples they plan to analyze.

2.3.1. 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.

2.3.2. 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.

2.3.3. 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.

2.3.4. 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.

2.3.5. 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 thiobarbituic acid solution and placing it onto pH paper.

2.3.6. 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.

3.1. 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.

3.2. 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.

3.3. 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**.

3.4. Take eight glass tubes (13 mm x 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.

4.1. 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.

4.2. 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.

4.3. Add 1.5 mL of the 3.5 M sodium acetate buffer (pH = 4) to each sample and standard.

4.4. Add 1.5 mL of the aqueous 0.8% thiobarbituric acid solution (pH = 4) to each sample and standard.

4.5. Bring the final volume to 4 mL for each sample and standard by adding 700 µL of DI water.

4.6. 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.

4.7. Remove the glass tubes from the heating block and incubate on ice for 30 min.

4.8. Centrifuge samples and standards at 1500 x 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.

4.9. Immediately after centrifugation, aliquot 150 µL of supernatant from each tube and place into a separate well of a 96 well plate.

4.10. 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.

4.11. Read absorbances at 532 nm. Subtract the average absorbance reading of the blank samples from all other absorbance readings.

4.12. 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.

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^{-6}, 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 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**).

FIGURE AND TABLE LEGENDS:

Figure 1: Thiobarbituric acid reactive substances assay schematic. 100 µL of sample or standard are added to a 13 mm x 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 x g for 10 min at 4 °C. 150 µL 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.

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.

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[21].

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.

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.

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 $CuCl_2$ (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.

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.

Table 2: Detection limits of the TBARS assay.

Table 3: Analytical reproducibility of TBARS in three different biological samples.

DISCUSSION:

Despite its limitations[1,3,4,7-9,10,12-14,15,19] 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[1,32-34]. 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 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 x 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.

DISCLOSURES:

The authors have no competing financial interests or other conflicts of interest to disclose.

ACKNOWLEDGMENTS:

The research reported here was supported in part by the National Cancer Institute of the National Institutes of Health under award no. R33 CA217702 and the Initiative for Maximizing Student Development (IMSD) program. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health.

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).

2. Ohkawa, H., N. Ohishi, K. Yagi. Reaction of linoleic acid hydroperoxide with thiobarbituric acid. *Journal of Lipid Research.* **19** (8), 1053-1057 (1978).
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).
4. Morales, M., Munné-Bosch, S. Malondialdehyde: Facts and artifacts. *Plant Physiology*. **180** (3)**,** 1246–1250 (2019).
5. Devasagayam, T. P. A., Boloor, K. K., Ramasarma, T. Methods for estimating lipid peroxidation: An analysis of merits and demerits. *Indian Journal of Biochemistry and Biophysics*. **40** (5), 300-308 (2003).
6. Dasgupta, A., Klein, K. Methods for Measuring Oxidative Stress in the Laboratory. In *Antioxidants in Food, Vitamins and Supplements*. 19-40 (2014).
7. Wade, C. R., van Rij, A. M. 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).
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).
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).
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).
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).
15. Jentzsch, A. M., Bachmann, H., Fürst, P., Biesalski, H. K. Improved analysis of malondialdehyde in human body fluids. *Free Radical Biology and Medicine*. **20** (2), 251–256 (1996).
16. Buege J. A., Aust, S. D. Microsomal lipid peroxidation. *Methods in Enzymology.* **52**, 302-310 (1978).
17. Gutteridge, J. M. C. 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).

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).
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).
20. Lee, R et al. "Evaluating oxidative stress in human cardiovascular disease: methodological aspects and considerations." *Current medicinal chemistry*. **19** (16), 2504-2520 (2012).
21. Morel, D. W., Hessler, J. R., Chisolm, G. M. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *Journal of Lipid Research*. **24** (8), 1070–1076 (1983).
22. Guzmán-Chozas, M., Vicario-Romero, I. M., 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*. **28 1**(2), 1196–1204 (2006).
24. Skoog, D. A., West, D. M., Holler, F. J., and Crouch, S. R. Sampling, standardization, and calibration. In *Fundamentals of Analytical Chemistry*. 9th ed., Ch. 7, 153-196, Cengage - Brooks/Cole, Belmont, CA, 2014.
25. Skoog, D. A., Holler, F. J., and Crouch, S. R. 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).
27. Jeffs, J. W. 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).
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, M. L. C., Wilbur, K. M. The reaction between thiobarbituric acid and the oxidation products of certain lipides. *Journal of Biological Chemistry.* **174** (1), 257-264 (1948).
30. Wilbur, K. M., Bernheim F., Shapiro, O. W. 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).
31. Kwon T. W., Watts, B. M. Determination of malonaldehyde by ultraviolet spectrophotometry. *Journal of Food Science.* **28** (6), 627-630 (1963).
32. Esterbauer H., Schaur F. J., Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biology & Medicine.* **11** (1), 81-128 (1991).
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).

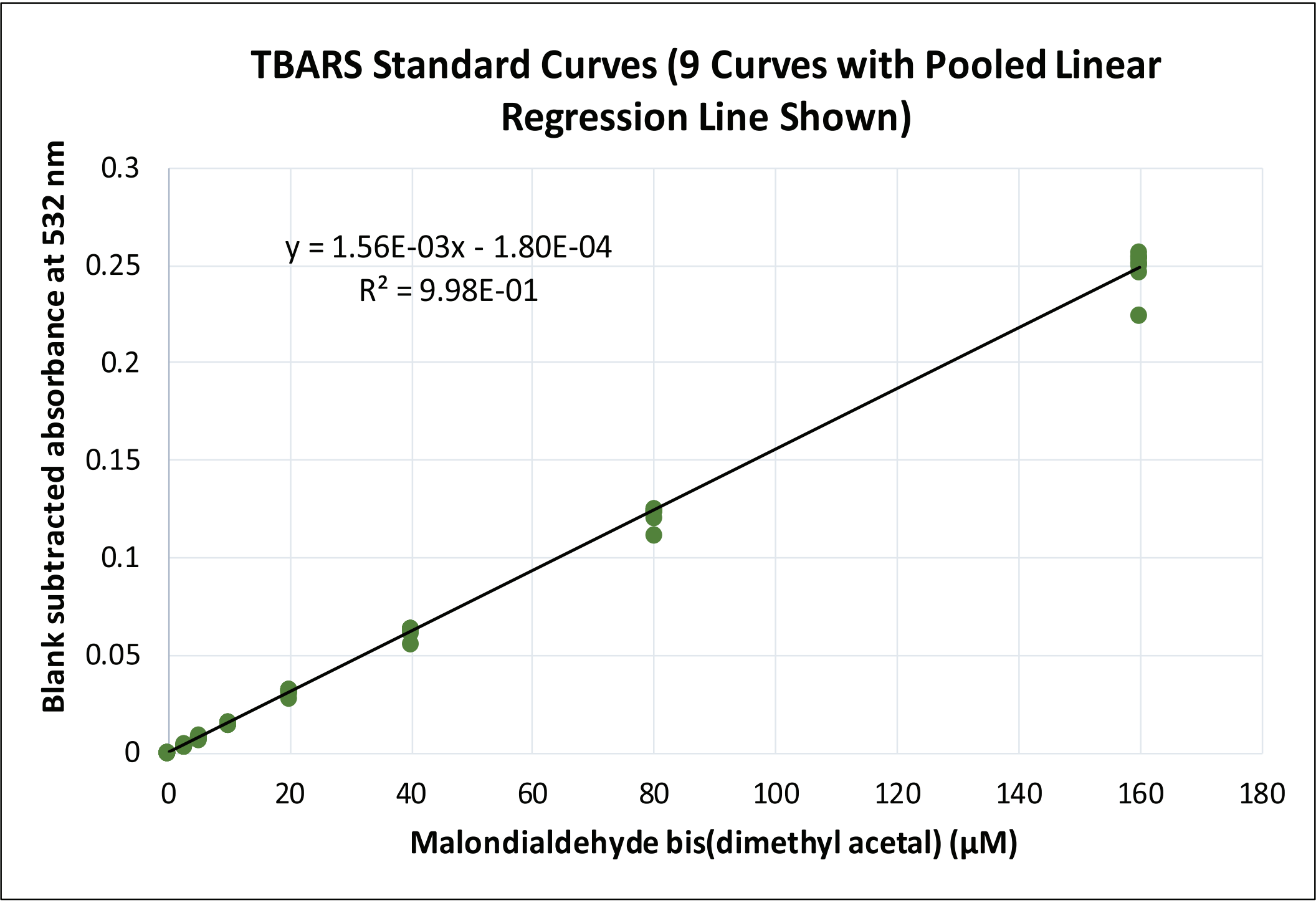
34. Jentzsch, A. M., Bachmann, H., Fürst, P., Biesalski, H. K. Improved analysis of malondialdehyde in human body fluids. *Free Radical Biology and Medicine*. **20** (2), 251–256 (1996).
35. Jo, C., Ahn, D. U. Fluorometric Analysis of 2-Thiobarbituric Acid Reactive Substances in Turkey. *Poultry Science*. **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).
37. Barden, A. E., Mas, E., Croft, K. D., Phillips, M., Mori, T. A. Minimizing artifactual elevation of lipid peroxidation products (F 2-isoprostanes) in plasma during collection and storage. *Analytical Biochemistry*. **449** (1), 129–131 (2014).
38. Jeffs, J. W., Ferdosi, S., Yassine, H. N., Borges, C. R. Ex vivo instability of glycated albumin: A role for autoxidative glycation. *Archives of Biochemistry and Biophysics*. **629**, 36–42 (2017).
39. Lee, D. M. Malondialdehyde in Stored Plasma. *Biochemical and Biophysical Research Communications*. **95** (4), 1663–1672 (1980).
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).

Figure

Figure

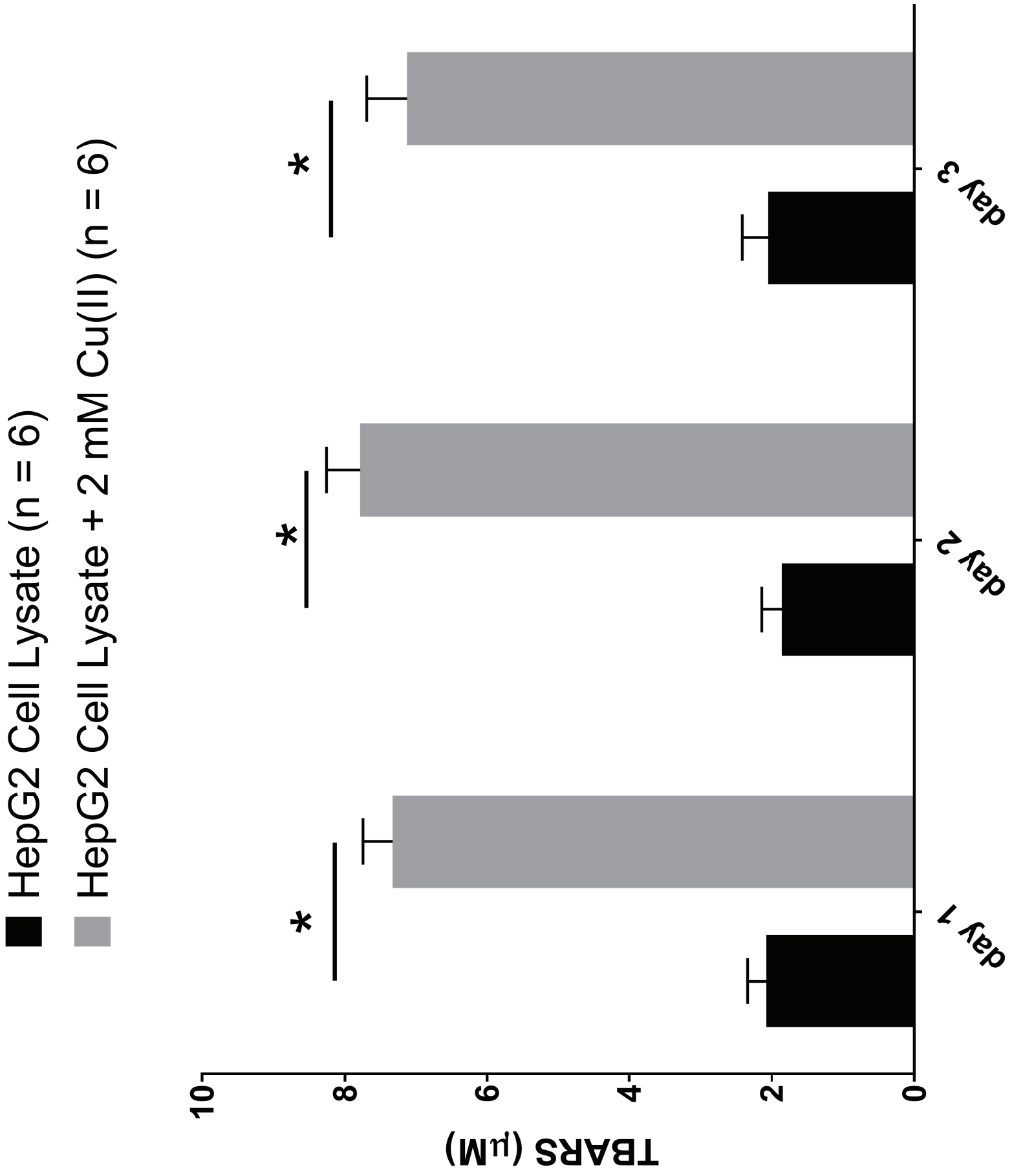
Figure

Click here to access/download;Figure;Figure 3.eps



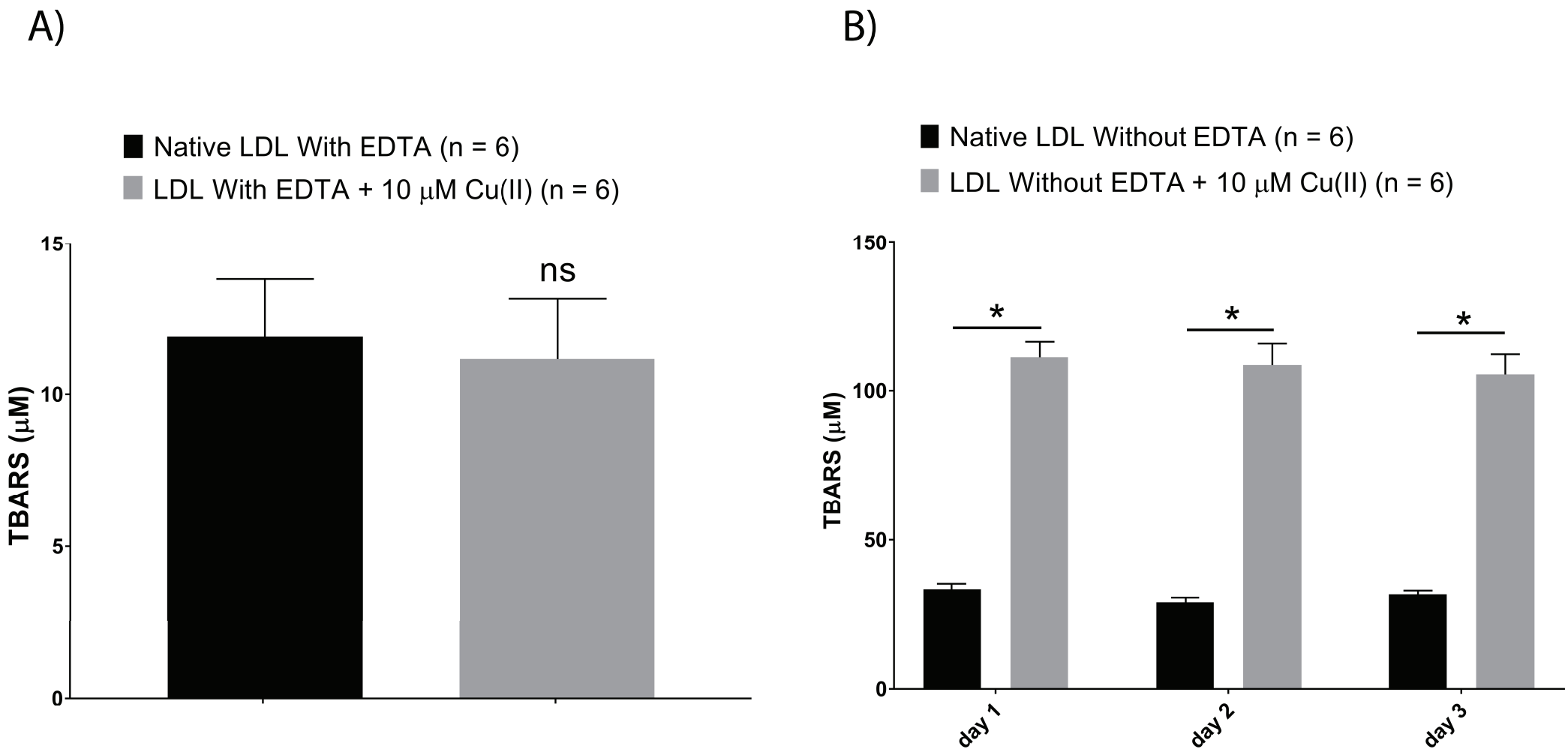
Figure

Click here to access/download;Figure;Figure 4_.eps



Figure

Click here to access/download;Figure;Figure 5.eps



Figure

Click here to access/download;Figure;Figure 6_.eps

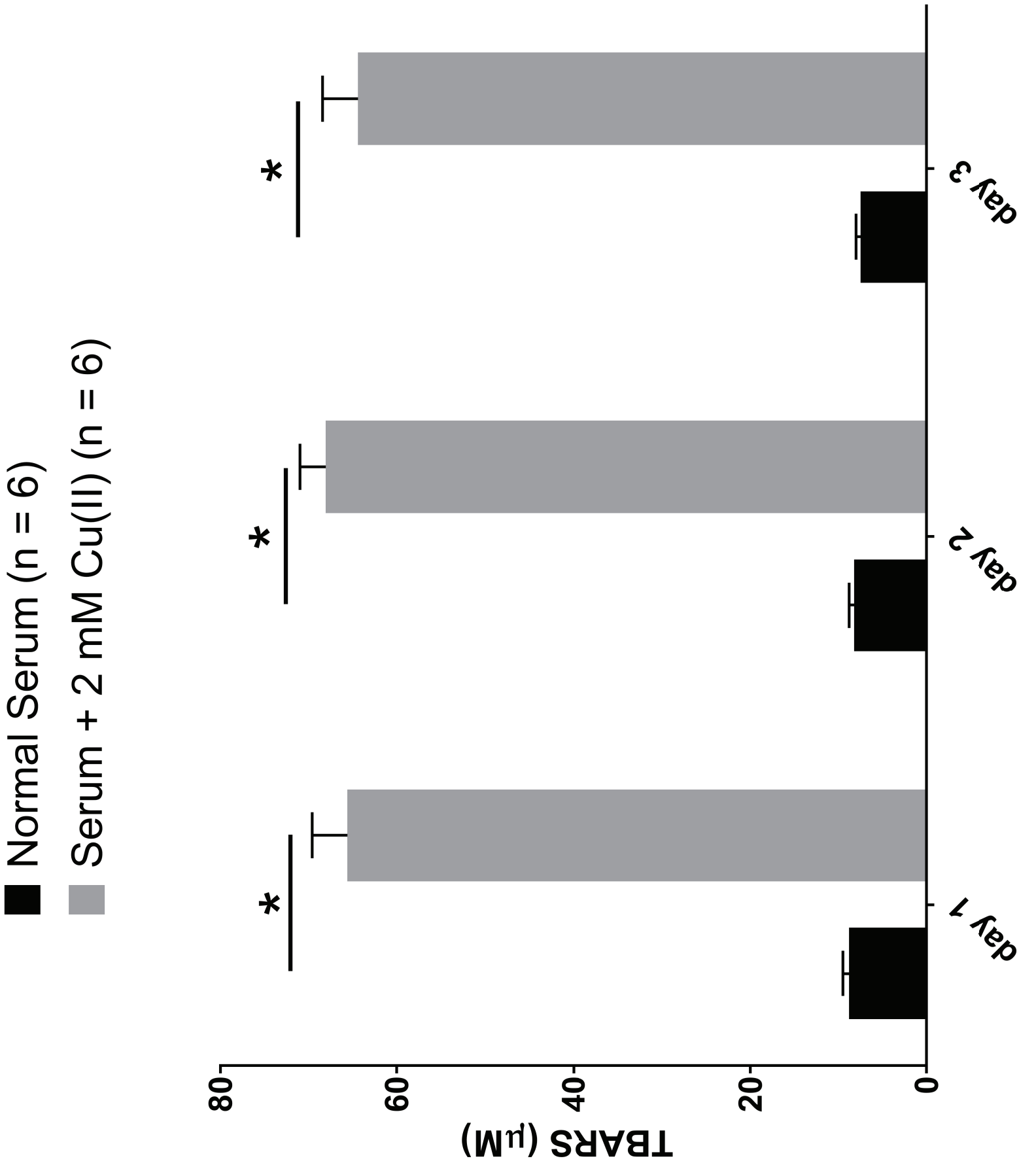


Table 1

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

Day	Absorbance [a]	S_{bl} [b]	S_m [c]	Sensitivity (absorbance units/µM)[d]
1 (n = 6)	0.0412	0.00061	0.0430	0.00160
2 (n = 6)	0.0415	0.00063	0.0433	0.00160
3 (n = 6)	0.0413	0.00061	0.0431	0.00160
All three days (n =	0.0413	0.00059	0.0431	0.00160

[a]Absorbance of the blank samples on three different days with 6 replicat

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

[c]S_m = Minimum distinguishable analytical signal, which was determined l plus a multiple k of the standard deviation of the blank (ks_{bl}), where k =

[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

c_m (µM)[e]
1.14
1.18
1.13
1.10

tes per day.

by summing the mean of the blank signal ($\bar{S}_{bl}$)
3. That is; $S_m = \bar{S}_{bl} + ks_{bl}$.

e m = the slope of the standard curve.

Table 3

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 $CuCl_2$		**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.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1x Sterile PBS pH 7.4 1 L	VWR, PA	101642--262	cell lysis reagent
50 mL self-standing centrifuge tube	Corning, NY	CLS430897	General material
96 well plate, Non-Treated, clear, with lid, Non-sterile	Thermo Fisher Scientific, MA	280895	To measure absorbance
Amicon Ultra-0.5 100 kD centrifugal spin filter device	Fisher Scientific, NH	UFC510024	LDL purification
Caps for glass tubes	Thermo Fisher Scientific, MA	14-930-15D	for TBARS assay
Copper II Chloride	SIGMA, MO	222011-250G	to induce oxidation
Culture tubes, Disposable, with Screw-Cap Finish, Borosilicate Glass (13 x 100 mm)	VWR, PA	53283-800	for TBARS assay
Eagle's Minimum Essential Medium (EMEM)	ATCC, VA	HB-8065	HepG2 cell media
Eppendorf Safe-Lock Tubes, 1.5 mL	eppendorf, NY	22363204	General material
Eppendorf Safe-Lock Tubes, 2.0 mL	Genesee Sceitific, CA	22363352	General material
Fetal Bovine Serum US Source	Omega Scientific, CA	FB-11	for cell culture
Glacial Acetic Acid	SIGMA, MO	27225-1L-R	TBARS Reagent
Halt Protease Inhibitor Cocktail (100x)	Thermo Scientific, MA	87786	cell lysis reagent
HEPES	SIGMA, MO	H3375-250G	LDL solvent
HepG2 Cells	ATCC, VA	HB-8065	Biological matrix prototype
Hydrocloric acid (HCl)	Fisher Scientific, NH	A144-212	cell lysis reagent
Legend Micro 17 Centrifuge	Thermo Scientific, MA	75002431	General material

Low Density Lipoprotein, Human Plasma	Athens Research & Technology, GA	12-16-120412	Biological matrix prototype
Magnetic Stir Bars, Octagon 6-Assortment	VWR, PA	58948-025	General material
Malondialdehyde bis (dimethyl acetal)	SIGMA, MO	8207560250	TBARS Standard
Multiskan Go Microplate Spectrophotometer	Fisher Scientific, NH	51119200	To measure absorbance
NP-40	EMD Millipore Corp, MA	492016-100ML	cell lysis reagent
Sodium Chloride	SIGMA, MO	S7653-1KG	cell lysis reagent
Sodium dodecyl sulfate (SDS)	SIGMA, MO	436143-100G	TBARS Reagent
Sodium hydroxide	SIGMA, MO	367176-2.5KG	TBARS Reagent
SpeedVac Concentrator	Thermo Scientific, MA	SC250EXP	For concentrating cell lysates
T-75 Flask, Tissue Culture Treated, 250 mL, w/filter cap	USA Scientific, FL	658175	cell culture
Thiobarbituric Acid	SIGMA, MO	T5500-100G	TBARS Reagent
TRIS base	Fluka, GA	93362	cell lysis reagent
Trypsin (1x)	VWR, PA	16777-166	To detach HepG2 cells

Author Responses to Reviewer Critiques

Thanks to the editor and reviewers for their efforts in critiquing this manuscript. Good points were raised which are now addressed in the revised manuscript as described in detail below in blue font. We feel the revisions suggested have improved the quality of the manuscript. All changes to the manuscript are shown in colored text in the "changes tracked" version of the manuscript. Please note that the line numbers referred to below correspond to the "changes tracked" version of the manuscript in which changes in Microsoft Word are shown "in line", not in balloons.

Editorial Comments

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author Response: We have proofread the manuscript and no spelling or grammar issues have been found.

2. Please provide at least 6 key words or phrases.

Author Response: The keyword "oxidative stress" has been added to make a total of 6 key words or phrases (line 20).

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Halt (protease inhibitor cocktail), Eppendorf, Amicon Ultra

Author Response: The words Halt, Eppendorf, Amicon Ultra, and Athens Research and Technology company phrase have been crossed out throughout the protocol and we used generic terms instead (lines 157, 168, 175, 206, 214, 225-226, 233, 258, 353). See manuscript with tracked changes.

Protocol:
1. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Author Response: We have added the following statement to lines 142-144 as requested: "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."

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Author Response: We added extra two steps to explain how to prepare the $CuCl_2$ stock solutions (see lines 184-190, 241-244). We've also added additional text to better explain how

to prepare the sodium acetate buffer solution pH 4 (lines 287-288, 294-295). See manuscript with tracked changes.

Figures:
1. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, or .pdf file (i.e., 5 files in total). Please remove 'Figure 1' etc. from the figures themselves.

Author Response: We have removed "Figure 1" etc. from the figures themselves and created individual files for each figure. There is a total of six figures (six files) now since we added an extra figure to address one of the reviewer comments. We have uploaded these as .eps files as instructed in the main body of the editor's email. We can convert them to PDF if needed.

Acknowledgment and Disclosures:
1. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Author Response: This has been done. See lines 573, 575.

References:
1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.
2. Please do not abbreviate journal titles.

Author Response: We have now added this format for the references, and corrected all journal title abbreviations (see references list).

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Author Response: We have added information on the rest of materials mentioned in the protocol. Also, we have added the state for the companies since this was requested by one of the reviewers. See Table of Materials, highlighted in blue.

Reviewer #1

The manuscript entitled "Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituic Acid Reactive Substances Assay" describes a well-known TBARS assay in biological samples. I know that it is a commonly used method in the researches dealing with oxidative stress and so many investigators were used it without enough attention on its reliability, reproducibility, repeatability, selectivity, sensitivity and other method validation criteria. The reported SD values for figures 3-5 looks excellent, however, we tried the assay using different methods from the literature and we have observed very large SD values. I regret to say that I cannot recommend its publication in your journal in its present form, until the authors convince the readers with acceptability of full validation data which could be found in the official guidelines of FDA and ICH. It is obvious that, ignoring the analytical method validation data will result in some misleading data, waste of time and resources.
It is interesting that the number of publications retrieved from Scopus using key words of "malondialdehyde" are 4631, 4906 and 144, in which 1527, 1460 and 24 of the articles

categorized in the subject area of "medicine", respectively for years 2018, 2019 and 2020. These figures reveal that MDA has an important position in biomedical investigations. However, in most of these reports researchers used usually TBARS assay without even partial validation tests. There are a number of publications dealing with the pitfalls of the assay and I am happy to review the authors' responses to these critics. As examples, I am listing some of them:
Clinical Chemistry 34, 208, 1988.
Clinical Chemistry 35, 336, 1989.
Bioimpacts, 5, 123-127, 2015.
Lung, 193, 867-868, 2015.
Clinical Oral Investigations, 20, 395-396, 2016.
Current Pharmaceutical Analysis, 12, 4-17, 2016.
Bioanalysis, 8, 2179-2181, 2016.
Pharmaceutical Sciences, 23, 193-197, 2017.
Annals of Surgical Oncology, 24, S573-S576, 2017.
Biomarkers in Medicine, 12, 551-554, 2018.
Plant Physiology 180, 1246-1250, 2019.

Author Response: We looked up and read the references cited by the reviewer. In short, we must conclude that the reviewer makes an excellent point. We had previously pointed out that as an analytical method the TBARS assay lacks specificity and can be impacted by pre-analytical specimen handling and storage. But thanks to this reviewer it has become very clear to us that the assay also lacks analytical ruggedness (i.e., the ability to make minor methodological modifications without significantly affecting assay results) to the point that we are now of the opinion that TBARS assay results should not be compared between laboratories in the absence of explicit inter-laboratory analytical validation data.

In conjunction with the recommendation made by Reviewer #2 to shorten the second two paragraphs of the Introduction, these have now been cut and replaced with new text (see lines 90-116 and 124-127 and 134) that describes some of the major issues surrounding the application and interpretation of the TBARS assay. In this section we state, among other things, that:

“Pre-analytical variables related to biospecimen handling and storage (e.g., blood plasma kept even temporarily at -20 °C)[14,15] can have a major impact on TBARS assay results [16,17]—so much so, that in our opinion, TBARS assay results should not be compared across different laboratories unless such a comparison is warranted by explicit inter-laboratory 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.” See lines 103-110.

We have also modified a few paragraphs in the Discussion section to align this new emphasis on correct analytical applicability with the Introduction and the rest of the paper (see lines 461-462, 481-485, 507-509, 512-513, 515-516). For example, we now start the first sentence of the Discussion with the statement, “Despite its limitations[1,3,4,7,9,10,12,14,15,19] and, in our opinion, lack of suitability for comparison between laboratories,…”. And we conclude the penultimate paragraph of the Discussion with the statement, “This means that even modest variability in the pre-analytical handling and storage of biospecimens that are to be compared to one another using the TBARS assay must be avoided.”

Finally, we would also like to respectfully point out that we did not "ignor[e] the analytical method validation data". On the contrary, we validated linearity, sensitivity and working dynamic range (now Fig. 3), limits of detection (Table 2), and intra- & interassay precision/reproducibility (Table 3). The latter was actually documented independently for each biological matrix analyzed as part of this manuscript (Table 3). As explained in the manuscript the assay is well known for its lack of selectivity. Because of this, we did not attempt to validate accuracy/recovery because without selectivity it is impossible to fully define accuracy. Moreover, even if accuracy/recovery were low (which others have proven it to be using a similar analytical standard to that employed in this study)[7], this would not matter as long as the assay was precise and interpretation was limited to relative intra-laboratory comparisons—which we now prescribe.

Given the limitations of the TBARS assay, it is surprising that anyone uses it today. As we describe now in the Introduction, "…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 enjoys today. Although there are other assays with greater analytical sensitivity, specificity and ruggedness used for the determination of oxidative stress in biological samples, because of its ease of use the TBARS assay based on absorbance at 532 nm still remains by far one of the most commonly used assays for the determination of lipid peroxidation[20], and thereby assessment of oxidative stress in general." (See lines 113-119.) This is the reason why we feel it is important to publish our revised manuscript: The unstoppable popularity of the TBARS assay in biomedical research requires papers like this one (and those cited by Reviewer #1) that put forward use-specific analytically validated methods alongside verbiage that includes caveats and warnings about the overall applicability and interpretability of the method.

Reviewer # 2

The authors provide economic, detailed, reproducible methodology for TBARS assay in low-density lipoproteins, cell lysates and human serum.

Author Response: We are pleased to see that, in general, the reviewer believes the method reported in the manuscript warrants publication.

1 - lanes 57-81: in my opinion, the background concerning diseases/pathologies and lipid peroxidation is not so appropriate and it lacks of important findings. However, the focus is a homemade assay that will be suitable for different research purposes that depend on researcher needs. The authors should rewrite lanes 57-81 in a concise way.

Author Response: As described above in our response to Reviewer #1, we have deleted lines 57-81 (along with a few additional lines), and instead used this space to re-orient the paper to more of an analytical focus, in which we describe how this assay should only be used for intra-laboratory experiments and that the data between labs are not compatible unless the assay has been explicitly validated as compatible between the labs (see revised Introduction section).

2 - Warnings should be written in a separate sections and preparations using a different character style. Laboratory preparations must be standardized by indicating the weighings and the volumes used for all ones. Abbreviations should be used correctly in all the manuscript.

Author Response: According to JoVE manuscript guidelines, the word “CAUTION” should be used to indicate any harmful or toxic chemicals being used in the protocol, in which the hazard and appropriate handling guidelines need to be described. We are not sure what type of character style is being asked for. We have now corrected all laboratory preparations and indicated the specific volumes and weights that were used in preparation of all reagents used in this assay. Specifically, for line 150 the volume of media used is indicated, for line 163 the volume of PBS is indicated, and so on. Correct abbreviations are indicated in lines 205-208, line 344-345, 348-350, 354, 405, line 407-409, and line 445.

3 - Schematic protocol/s should be added.

Author Response: We have added a new graphics/photos-based figure (Figure 1) showing the workflow schematic of the protocol used here. See Figure 1, and the new caption for Figure 1.

4 - When ELISA or colorimetric assays are developed an accurate and easier volumes are preferred. Why 94 µl of sample and not 95 µl? Why 6 µl of sample and not 5 µl? Why 24 µl of sample and not 25 µl? And so on. The authors should calculated the stock solution concentration to make the pipetting easier.

Author Response: Due to the fact that adjustable-volume air displacement pipettes are ubiquitous features of nearly every modern research laboratory, we feel that it is neither easier or more difficult to pipette 94 vs. 95 µL or 6 vs. 5 µL, etc. As such, we did not take this consideration into account when conducting the experiments for this manuscript. Because of this, we cannot include the altered volumes in the protocol because they are not what we used. We would have to repeat every experiment with slightly altered volumes and concentrations to fulfill this request, which is an undertaking that we did not feel was scientifically necessary—especially given the universality of adjustable volume pipettes.

5 - The authors should, more or less, specify the cost to test 96 samples to demonstrate the lower cost of their assays than that of a commercial kit

Author Response: Good point. For 100 grams of thiobarbituric acid (TBA) the cost is $105 dollars, and here we prepared 500 ml of 0.8 % TBA solution, for which we only used 4 grams of TBA. However, for 96 samples only 144 ml of the 0.8 % TBA solution are needed. Hence, about $1.21 for thiobarbituric acid is needed for 96 samples. The cost for 250 ml of MDA bis dimethyl acetal is $65 dollars, and we only used 92 µl for a 1000 ml stock solution. Hence, the cost for MDA standard is insignificant (about $0.0239 dollars) for 96 samples. For preparing sodium acetate buffer we used 100 ml of glacial acetic acid and 13 grams of sodium hydroxide beads. The cost for 1000 ml of acetic acid is $74 dollars, and the cost for 2500 grams of sodium hydroxide beads is $102 dollars. However, for 96 samples, only 144 ml of sodium acetate buffer pH 4 are needed. Hence the total cost for the sodium acetate buffer solution pH 4 for 96 samples is about $0.0317 dollars. Finally, in this protocol we prepared excess 8.1 % SDS solution since only 200 µl are needed per sample and we prepared 400 ml. For 96 samples, about 20 ml of 8.1 % SDS solution are needed, in which 1.62 grams are needed to prepare such solution. The price for 100 grams of SDS is $141 dollars, about $1.4 dollars per gram and therefore $2.27 for 96 samples. Therefore, the total cost to test 96 samples using our assay is about $3.5 dollars, extremely low when compared to the commercial kit which costs about $400

dollars. This has been indicated in abstract and discussion sections, specifically lines 44 and 466-467.

6 - The authors should argument better the concept of the sentence in lanes 98-99 as, for some research laboratories, it is not a problem to test about 25 different samples all together (one for each experimental condition repeated 3 times = 75) in a day with only one standard curve

Author Response: For researchers that consistently run nearly 96 samples in a batch and therefore make efficient use of their purchased TBARS assay kits, this is not a problem. But for other researchers it can represent a problem when they intend to determine the levels of oxidation in a biological sample at different time points. We have added an extra sentence (line 124-127) to better explain this concept.

7 - The authors have chosen cell lysates of HepG2, but they not specify if their assay could be used for all cell lysates

Author Response: HepG2 cell lysates are a prototype cell lysate used in this assay. Strictly speaking, assay performance characteristics would have to be validated for other cell types (i.e., other biological matrices), but based on the results presented here, it is reasonable to expect that the assay will work with most other types of cell lysate. We have added this information in lines 431-435.

8 - Is it possible using other lysis buffer instead of RIPA buffer?

Author Response: We chose RIPA buffer because it is commonly utilized to lyse cells. The general ingredients for most lysis buffers are buffer, NaCl, MgCl, KCl, protease inhibitors and detergents. It is expected that most other lysis buffers will not interfere with the TBARS assay. However, if researchers choose to use a different lysis buffer or add additional ingredients, preliminary validation studies should be conducted to make sure the lysis buffer of choice is compatible with the TBARS assay used here. We have added this information in lines 159-161.

9 - Is it possible detach cells with trypsin 1X?

Author Response: When working with adherent cells, there are two general ways of performing a lysis procedure; one way is to add the lysis buffer directly to detach and lyse the cells at the same time, and alternate way is by detaching the cells with trypsin to collect cell pellets for lysis. When it comes to choosing either procedure, it is a researcher's choice, and there is not a reason to believe that one method would work better than the other. Here we chose to add the lysis buffer directly to the cells. However, It is also possible to detach the cells first with trypsin 1X, wash the cell pellets with PBS, and then reconstitute each cell pellet with lysis buffer. The main goal of this manuscript is to show the applicability of the TBARS assay in different biological matrices, and it is difficult to cover every possible way to do a general cell lysis procedure, which was not the main goal of the manuscript. However, based on the results obtained here, it is reasonable to assume that detaching the cells with trypsin will also work. If trypsin is used and then media employed to neutralize it, cell pellets should be washed twice with PBS prior to adding the lysis buffer. We added this information in lines 176-180.

10 - Is it always mandatory concentrate the cell lysate supernatant? Is it possible diminish the volume of lysis buffer not to concentrate the sample?
Author Response: No, it is not mandatory to concentrate the cell lysate supernatant. It is possible to diminish the volume of lysis buffer to avoid concentration of the sample. Here we chose to add more lysis buffer to cover all the cell surface in a T75 flask. However, if the cells are detached with trypsin, cell pellets can be reconstituted/lysed with a lower volume of lysis buffer, in this case 250 µl of lysis buffer in each cell pellet. It is recommended to have a high concentration of cell lysate to increase the analytical signal and therefore the precision of the TBARS assay. We have added this information in the manuscript with tracked changes lines 176-180.

11 - Please, specify the volume of each sample for unknown samples and for standard curve (100 or 150 µl?)

Author Response: The volumes for unknown samples and for each standard should be 100 µl. We have specified this better in the sample preparation section (pg. 7). We have specifically deleted part of the sentence in line 252 to avoid confusion. A 100 µl final sample volume is now clearly specified in line 367.

12 - Please, specify better in Figure 4a that the LDL samples contain EDTA

Author Response: We specify in the figure caption that LDL samples in Figure 4a (now Figure 5a) contain EDTA, but we have added additional information in Figure 5a and 5b to better specify the difference between samples containing EDTA and the ones that do not contain EDTA (see Figure 5 and corresponding caption).

13 - $p<0.001$, one * is enough if Figures

Author Response: we have corrected this in all figures containing significant differences to show only one *.

14 - Please, provide the State for the companies

Author Response: We have added the State or residence for the companies in the Table of Materials since according to editor comments we are not allowed to use commercial language and company names in the manuscript. See Table of Materials.

Reviewer # 3

The manuscript "Evaluation of oxidative stress in biological samples using the thiobarbituric acid reactive substance assay" describes study in which detailed protocol for determination of MDA is described.
As author stressed, the thiobarbituric assay is widely used method for determination of oxidative stress in biological samples. If properly conducted reliable results could be obtained. Described protocol is not new but gives detailed procedure. The detailed procedure is always needed, and each analyst will adopt the method that is the most suitable for its type of sample (and budget).

Author Response: We are pleased to see that, in general, the reviewer believes the method reported in the manuscript warrants publication.

Minor Concerns:
I have only one question. Can the authors explain why they omitted use of BHT (that is usually added to samples to prevent additional oxidation of sample).

Author Response: BHT is a radical scavenger that can help to minimize artefactual formation of TBARS in order to preserve the "natural" biological state of a biospecimen. In this study we were not interested in studying biological processes per se, but rather we were interested in documenting the performance characteristics of a basic analytical method.

As documented by the comments of Reviewer #2 and our responses to them, there are a great many possible methodological variations that could be applied to this assay and we could not possibly have tested or validated all of them. This manuscript provides a useful but general protocol that can be further adapted (and validated) for specific applications—including those for which the addition of BHT to samples is appropriate or mandated. We have now added this information to the Discussion (lines 481-485).

Reviewer # 4
The manuscript explain and discuss a popular method in biochemical research i.e. TBARS assay. The article is designed an written very well and contain all necessary information about the importance and experimental details of MDA measurement.
It contains the good information about usage of this method in different biological samples.

Author Response: We thank the reviewer for his/her positive remarks.

Minor Concerns:
In legend of figures * is set for showing statistical significance; however in the figures the *** symbol is used.

Author Response: We have now corrected this, and both the figure captions and graphs consistently show one *.

Just a question : there any other compound that can be used as standard? if it ai suggest to point out in the article (just the name)

Author Response: For this assay, only malondialdehyde (MDA) should be used as a standard—but pure, authentic MDA is not stable and is not commercially available. However, different chemical forms of MDA are commercially available such as, MDA tetrabutylammonium salt, MDA bis (dimethyl acetal), and MDA bis (diethyl acetal). Of these three forms of MDA, we used MDA bis (dimethyl acetal) since the vast majority of studies have used this same standard[21,22]. It has been demonstrated that the acidic conditions of the assay allow the release of MDA from MDA bis (dimethyl acetal)[1]; MDA then reacts with thiobarbituric acid (TBA) to form MDA-TBA_2 adducts that give a pink color. We did not test the other two chemical forms of MDA that are commercially available, however if researchers plan to use the other two forms of MDA as a standard, they should do so prior testing (see the new text addressing this question in lines 337-342).