Parameter™

TBARS Assay

Catalog Number KGE013

For the quantitative determination of Thiobarbituric Acid Reactive Substances (TBARS) concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Oxidizing agents can alter lipid structure, creating lipid peroxides that result in the formation of malondialdehyde (MDA), which can be measured as Thiobarbituric Acid Reactive Substances (TBARS) (1-3). First used in 1978, the measure of TBARS is still a commonly used and convenient method of determining the relative lipid peroxide content of sample sets, including serum, plasma, urine, cell lysates and cell culture supernates (1-3). Lipids that are multi-unsaturated (three or more double bonds) are both most likely to form peroxides and the most reactive in the TBARS assay (3-5). Free MDA is typically quite low, requiring release of MDA by acid treatment of proteins and breakdown of peroxides by heat and acid to facilitate color development in the TBARS reaction (3, 5-7). Removal of protein by precipitation eliminates potentially interfering amino acids that may react with thiobarbituric acid (3, 7).

Historically, TBARS assay methods have included variations in sample collection, storage, acidification and derivatization, protein clearance, standardization, correction for background sample absorbance, and detection by absorbance, fluorescence, or HPLC (3-10). These variations may cause difficulty when attempting to correlate values from one study to another. This assay is best used to compare one set of samples to another using a single method (3, 5, 10). TBARS measurements in serum or plasma are reported to increase modestly with age, and may be affected by dietary vitamins C and E, saturation of dietary fats, and concentration of triglycerides (2, 3, 11). The presence of biliverdin in icteric serum or erythrocyte membrane lipids in hemolyzed serum is reported to affect TBARS measurements (5, 6, 8).

Plasma TBARS measurements have been reported to correlate with some clinical features of cardiovascular disease, sepsis, preeclampsia, ischemia/reperfusion, chronic obstructive pulmonary disease, chronic pancreatitis, chronic kidney disease, cerebrovascular disorders, and bipolar disorder (2, 3, 11-13). Smoking is reported to increase TBARS in plasma (5, 10, 11). This assay has also been used to assess cell sensitivity to oxidants or antioxidant qualities of foods and additives *in vitro* (4, 14-16).

The Parameter TBARS assay is a 2-3 hour chemical analysis designed to measure TBARS in cell culture supernates, cell lysates, serum, plasma, and urine.

PRINCIPLE OF THE ASSAY

In the presence of heat and acid, MDA reacts with TBA to produce a colored end product that absorbs light at 530-540 nm. The intensity of the color at 532 nm corresponds to the level of lipid peroxidation in the sample. Unknown samples are compared to the standard curve.

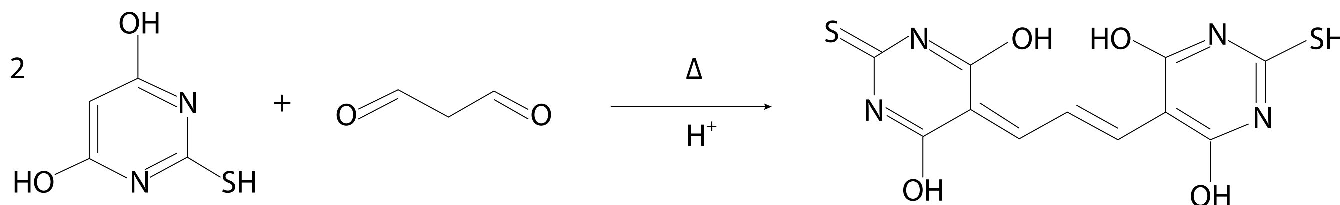


Figure 1: In the presence of acid and heat two molecules of 2-thiobarbituric acid (TBA) react with MDA to produce a colored end product that can be easily quantified.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with deionized or distilled water and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Reaction temperature should be in the range of 45-50 °C. Incubation temperatures under 45 °C will take longer than 3 hours to reach equilibrium, while temperatures over 50 °C will reach equilibrium in less than 2 hours.

PRECAUTION

TBARS Acid Reagent, trichloroacetic acid, is corrosive and causes severe skin burns and eye damage. It is very toxic to aquatic life with long lasting effects. Do not breathe dusts or mists. Wash hands thoroughly after handling. Wear protective gloves/protective clothing/eye and face protection. Avoid release to the environment. Refer to the MSDS for handling and disposal instructions.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Plate, Uncoated	892880	Two 96 well microplates (12 strips of 8 wells).	Return unused wells to the foil pouch and reseal along entire edge of the zip-seal. Store at room temperature.*
TBA Reagent	894140	15 mL of thiobarbituric acid in an aqueous solution. *May contain a precipitate. Place in a hot water bath and mix until completely dissolved.*	May be stored for up to 1 month at 2-8 °C.*
TBARS Standard	894141	1 mL of 500 µM 1,1,3,3-tetramethoxypropane in deionized water.	
TBARS Acid Reagent	895977	2 vials (15 mL/vial) of 0.6 N trichloroacetic acid in deionized water.	
Plate Sealers	N/A	4 adhesive strips	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 530-532 nm.
- 45-50 °C incubator.
- Microcentrifuge capable of ≥ 12,000 x g.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Microcentrifuge tubes for acid treatment.
- Test tubes for dilution of standards.
- TBARS Controls (optional; available from R&D Systems).

OTHER SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Cell Lysis Buffer (R&D Systems, Catalog # 895366).
- PBS

Or:

- Sonicator
- PBS

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Prior to assay, cells must be lysed according to the directions in the Cell Lysis Procedure section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Icteric and hemolyzed samples are not suitable for use in this assay.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

CELL LYSIS PROCEDURE

Use either of the following procedures for the preparation of cell lysate samples.

1. Perform a 5-fold dilution of Cell Lysis Buffer 3 with deionized or distilled water.
2. Wash cells two times in cold PBS.
3. Resuspend cells at 1 x 10^6 cells/mL in diluted Cell Lysis Buffer 3.
4. Incubate with gentle agitation for 30 minutes at 2-8 °C and freeze/thaw cells once at ≤ -20 °C.
5. Cell lysates do not need centrifugation prior to acid treatment.

Alternatively:

1. Wash cells one time in cold PBS.
2. Resuspend cells at 1 x 10^6 cells/mL in deionized water.
3. Sonicate for 10 seconds and freeze/thaw cells at ≤ -20 °C.
4. Repeat step 3 two times for a total of three sonication and freeze/thaw cycles.
5. Cell lysates do not need centrifugation prior to acid treatment.

ACID TREATMENT

All samples require acid treatment prior to assay. This clarifies the samples by precipitating interfering proteins and other substances for removal by centrifugation, and also catalyzes the TBARS reaction.

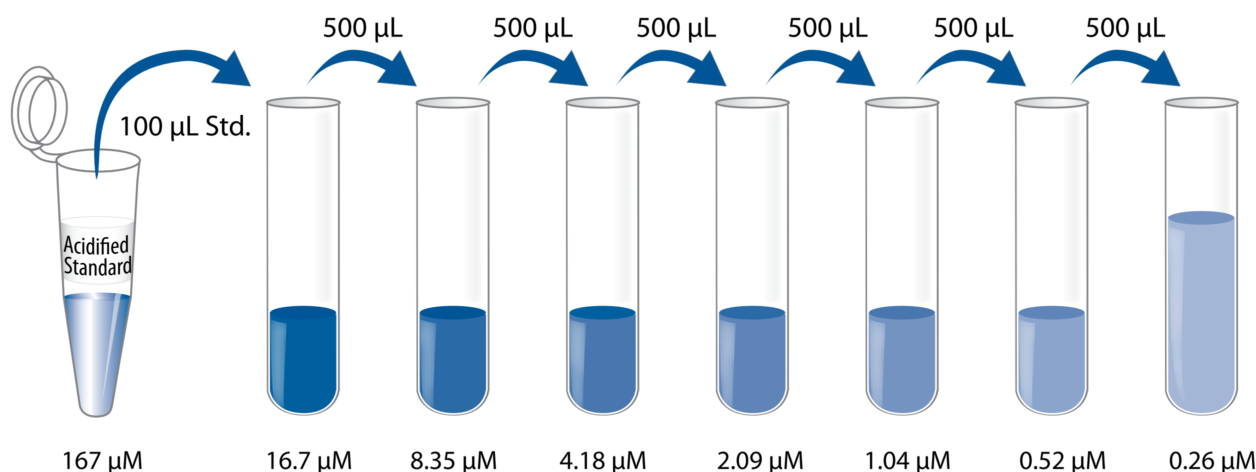
1. Add 300 µL of sample and 300 µL TBARS Acid Reagent to a microcentrifuge tube and mix well.
2. Incubate for 15 minutes at room temperature.
3. Centrifuge at ≥ 12,000 x g for 4 minutes.
4. Carefully remove and retain the supernate.
5. Repeat steps 3 and 4 if necessary to remove any remaining particulate. Assay immediately.
6. The concentration read off the standard curve must be multiplied by the dilution factor, 2.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

TBARS Standard - Convert the standard to MDA by adding 100 µL of TBARS Standard to 200 µL of TBARS Acid Reagent. Allow the standard to sit for a minimum of 30 minutes with gentle agitation. This produces a stock solution of 167 µM.

Pipette 900 µL of deionized water into the 16.7 µM tube. Pipette 500 µL of deionized water into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 16.7 µM standard serves as the high standard and deionized water serves as the 0 µM standard.



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, TBARS standard, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 150 µL of standards and samples* to each well.
4. Add 75 µL of TBA Reagent to each well.
5. Pre-read the optical density of each well using a microplate reader set to 532 nm.
6. Cover with the adhesive strip provided and incubate the microplate for 2-3 hours at 45-50 °C.
7. Determine the optical density of each well using a microplate reader set to 532 nm. Subtract pre-reading from the final reading to correct for the sample's contribution to the final absorption at 532 nm.

*Samples, standards, and controls require acid treatment or lysis and acid treatment. See Cell Lysis Procedure and Acid Treatment sections.

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CALCULATION OF RESULTS

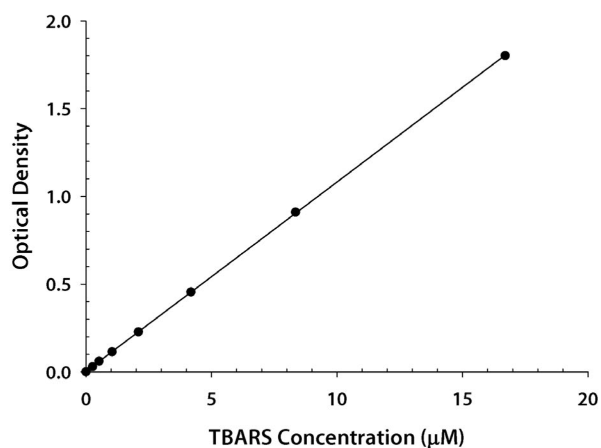
Subtract the optical densities (O.D.) obtained for each standard, sample and control prior to the incubation with the TBA Reagent from the O.D. for the same wells after incubation. Average the corrected duplicate readings for each standard, control, and sample.

Create a standard curve by reducing the data using computer software capable of generating a linear curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a linear x-axis and draw the best fit line through the points on the graph. Include the zero standard in the standard curve.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(µM)	O.D.	Pre-read O.D.	Corrected	Average
0	0.033	0.031	0.002	0.001
	0.035	0.034	0.001	
0.26	0.062	0.032	0.030	0.029
	0.063	0.034	0.029	
0.52	0.090	0.032	0.058	0.061
	0.099	0.035	0.064	
1.04	0.147	0.035	0.112	0.114
	0.147	0.031	0.116	
2.09	0.256	0.034	0.222	0.228
	0.266	0.033	0.233	
4.18	0.488	0.032	0.455	0.455
	0.489	0.033	0.457	
8.35	0.943	0.033	0.910	0.910
	0.944	0.034	0.910	
16.7	1.831	0.035	1.796	1.802
	1.841	0.034	1.807	

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (μM)	2.57	6.07	9.26	2.39	5.71	8.76
Standard deviation	0.025	0.078	0.109	0.125	0.242	0.322
CV (%)	1.0	1.3	1.2	5.2	4.2	3.7

RECOVERY

The recovery of MDA spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range %
Cell culture media (n=4)	101	94-110
Cell Lysis Buffer 3 (n=1)	99	96-103
Serum (n=4)	98	87-105
EDTA plasma (n=4)	91	89-106
Heparin plasma (n=4)	99	94-108
Urine (n=4)	99	91-106

*Samples were acid treated prior to assay. See the Acid Treatment section.

SENSITIVITY

Twenty-six assays were evaluated and the minimum detectable dose (MDD) of TBARS ranged from 0.007-0.055 μM. The mean MDD was 0.024 μM.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of MDA, acid treated, and serially diluted with deionized water to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell Lysis Buffer 3 (n=1)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	98	101	104	103	100	102
	Range (%)	95-99	—	101-108	101-105	96-103	99-105
1:4	Average % of Expected	97	101	102	100	98	100
	Range (%)	91-99	—	101-102	96-104	92-102	94-105
1:8	Average % of Expected	97	101	102	102	98	99
	Range (%)	94-99	—	97-105	98-105	92-103	92-105
1:16	Average % of Expected	95	98	100	103	100	99
	Range (%)	93-98	—	93-113	100-108	92-109	95-104

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of TBARS in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µM)	Range (µM)	Standard Deviation (µM)
Serum* (n=38)	0.280	0.038-0.929	0.215
EDTA plasma* (n=38)	0.285	0.006-0.974	0.218
Heparin plasma* (n=38)	0.264	0.005-0.992	0.215
Urine* (n=20)	1.08	0.440-2.81	0.550

*Samples were acid treated prior to assay. See the Acid Treatment section.

Cell Culture Supernates - Human peripheral blood lymphocytes (PBLs) were cultured in DMEM supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed, acid treated, and assayed for levels of TBARS. All samples had non-detectable levels of TBARS.

Cell Lysates - HeLa human cervical epithelial carcinoma cells were cultured in RPMI and supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and incubated for 6 days at 37 °C. Cells were untreated or stressed with 100 µM iron(II) sulfate for 1 hour at 37 °C. Cells were lysed with the sonication method described in the Cell Lysis Procedure section. Aliquots of the lysates were assayed for levels of TBARS. No detectable levels were observed

SPECIFICITY

Preparations of the following potentially interfering substances were prepared in PBS and PBS with ~6 µM MDA and assayed for interference. No significant interference was observed.

Antipain
Chymostatin
Leupeptin
PMSF
ProClin® 300
Sodium Azide
Sucrose
Trypsin Inhibitor
Triton™ X-100
Tween® 20
Tween® 80

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