

Antioxidant Assay Kit

Item No. 709001

TABLE OF CONTENTS

GENERAL INFORMATION	3	Materials Supplied
	4	Precautions
	4	If You Have Problems
	4	Storage and Stability
	4	Materials Needed but Not Supplied
INTRODUCTION	5	Background
	5	About This Assay
PRE-ASSAY PREPARATION	7	Reagent Preparation
	8	Sample Preparation
ASSAY PROTOCOL	10	Plate Set Up
	12	Performing the Assay
ANALYSIS	13	Calculations
	14	Performance Characteristics
RESOURCES	15	Interferences
	16	Troubleshooting
	16	Additional Reading
	16	References
	17	Related Products
	18	Warranty and Limitation of Remedy
	19	Plate Template
	20	Notes

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
10004872	Antioxidant Assay Buffer (10X)	1 vial
10004873	Antioxidant Assay Chromogen	3 vials
10004875	Antioxidant Assay Metmyoglobin	2 vials
10004876	Antioxidant Assay Trolox	3 vials
10004877	Antioxidant Assay Hydrogen Peroxide	1 vial
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.



WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

It is recommended to take appropriate precautions when using the kit reagents (i.e., lab coat, gloves, eye goggles, etc.) as some of them can be harmful.

Hydrogen Peroxide is corrosive and is harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes. Keep away from combustible materials.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at 4°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 750 nm or 405 nm; *NOTE: The absorbance may be read at 405 nm; however, there is less interference at 750 nm*
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism. Unstable free radical species attack cellular components causing damage to lipids, proteins, and DNA which can initiate a chain of events resulting in the onset of a variety of diseases.¹ Living organisms have developed complex antioxidant systems to counteract ROS and to reduce their damage. These antioxidant systems include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of small molecules, including ascorbic acid, α -tocopherol, β -carotene, reduced glutathione, uric acid, and bilirubin. The sum of endogenous and food-derived antioxidants represents the total antioxidant activity of the system. The cooperation among different antioxidants provides greater protection against attack by reactive oxygen or nitrogen species, than any single compound alone. Thus, the overall antioxidant capacity may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and body fluids.

About This Assay

Cayman's Antioxidant Assay can be used to measure the total antioxidant capacity of plasma, serum, urine, saliva, or cell lysates. Aqueous- and lipid-soluble antioxidants are not separated in this protocol, thus the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathione, uric acid, etc. are assessed (see Figure 1 on page 6). The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS[®] (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS^{••+} by metmyoglobin. The amount of ABTS^{••+} produced can be monitored by reading the absorbance at 750 nm or 405 nm. Under the reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 750 nm or 405 nm to a degree which is proportional to their concentration.²⁻⁵ The capacity of the antioxidants in the sample to prevent ABTS[®] oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents.

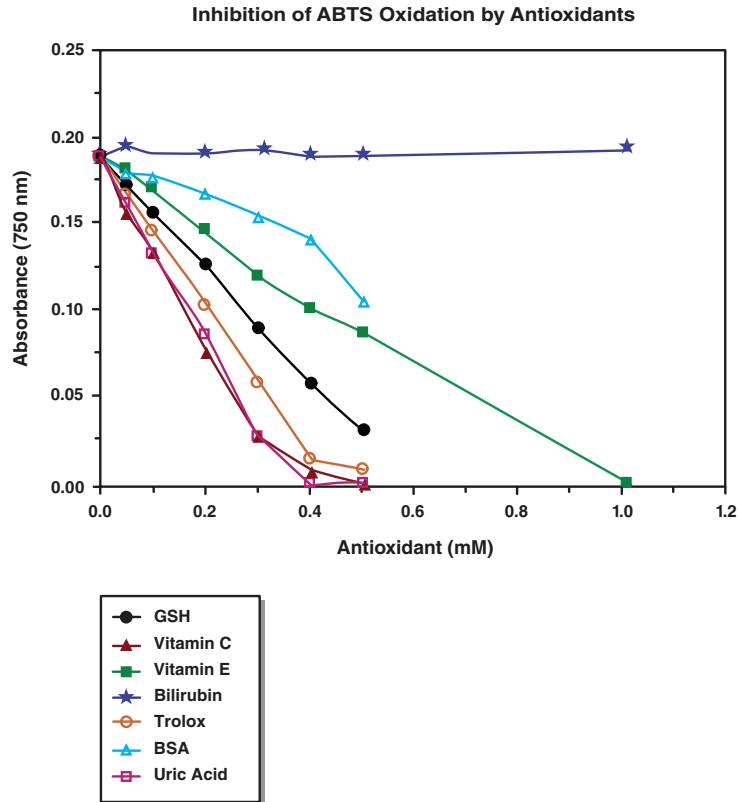


Figure 1. Inhibition of ABTS oxidation by antioxidants

Reagent Preparation

Some of the kit components are in lyophilized (under vacuum) or concentrated form and need to be reconstituted or diluted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used. For lyophilized components, stoppers must be removed slowly to allow air to enter the vials gradually, thereby preventing loss of material.

1. Antioxidant Assay Buffer (10X) - (Item No. 10004872)

Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This diluted Assay Buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) should be used to reconstitute the metmyoglobin. *NOTE: The use of 'Assay Buffer' in the remainder of the instructions refers to the diluted buffer.* When stored at 4°C, this Assay Buffer is stable for at least six months.

2. Antioxidant Assay Chromogen - (Item No. 10004873)

These vials contain a lyophilized powder of ABTS[•]. Reconstitute the Chromogen by adding 6 ml of HPLC-grade water to the vial and vortex well. One reconstituted vial will be sufficient for 40 wells. Reconstitute only the number of vials needed to assay the standards and samples. The reconstituted reagent is stable for 24 hours at 4°C.

3. Antioxidant Assay Metmyoglobin - (Item No. 10004875)

These vials contain a lyophilized powder of metmyoglobin. Reconstitute the metmyoglobin by adding 600 µl of Assay Buffer to the vial and vortex well. One reconstituted vial will be sufficient for 60 wells. The reconstituted reagent is stable for at least one month if stored at -20°C.

4. Antioxidant Assay Trolox - (Item No. 10004876)

These vials contain a lyophilized powder of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Reconstitute the Trolox vial by adding 1 ml of HPLC-grade water and vortexing well. The reconstituted reagent is used to prepare the Trolox standard curve. The reconstituted reagent is stable for 24 hours at 4°C.

5. Antioxidant Assay Hydrogen Peroxide - (Item No. 10004877)

This vial contains an 8.82 M solution of hydrogen peroxide. Dilute 10 μ l of hydrogen peroxide with 990 μ l of HPLC-grade water. Further dilute by removing 20 μ l and diluting with 3.98 ml of HPLC-grade water to yield a 441 μ M working solution. The Hydrogen Peroxide Working Solution is stable for four hours at room temperature.

Sample Preparation

Plasma

Typically, human plasma has an antioxidant capacity of 0.5-2 mM.^{2,5-7}

1. Collect blood using an anticoagulant such as heparin or citrate. Do not use EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.
3. Plasma should be diluted 1:20 or 1:30 with Assay Buffer before assaying.

Serum

Typically, human serum has an antioxidant capacity of 0.5-2 mM.^{8,9}

1. Collect blood without using an anticoagulant such as heparin or citrate. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
3. Serum should be diluted 1:20 or 1:30 with Assay Buffer before assaying.

Urine

Typically, human urine has an antioxidant capacity of 0.2-3 mM.⁹

1. Collect urine in a clean beaker or flask and store on ice. If not assaying on the same day, freeze the sample at -80°C.
2. Urine should be diluted 1:10 or 1:20 with Assay Buffer before assaying.

Saliva

Typically, human saliva has an antioxidant capacity of 0.3-1 mM.⁹

1. Collect saliva in a clean beaker or flask and store on ice. If not assaying on the same day, freeze the sample at -80°C.
2. Saliva should be diluted 1:2 with Assay Buffer before assaying.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. The cell pellet can be homogenized or sonicated on ice in 1-2 ml of cold buffer (*i.e.*, 5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of Trolox standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	(A)	(A)	(S2)	(S2)	(S10)	(S10)	(S18)	(S18)	(S26)	(S26)	(S34)	(S34)
B	(B)	(B)	(S3)	(S3)	(S11)	(S11)	(S19)	(S19)	(S27)	(S27)	(S35)	(S35)
C	(C)	(C)	(S4)	(S4)	(S12)	(S12)	(S20)	(S20)	(S28)	(S28)	(S36)	(S36)
D	(D)	(D)	(S5)	(S5)	(S13)	(S13)	(S21)	(S21)	(S29)	(S29)	(S37)	(S37)
E	(E)	(E)	(S6)	(S6)	(S14)	(S14)	(S22)	(S22)	(S30)	(S30)	(S38)	(S38)
F	(F)	(F)	(S7)	(S7)	(S15)	(S15)	(S23)	(S23)	(S31)	(S31)	(S39)	(S39)
G	(G)	(G)	(S8)	(S8)	(S16)	(S16)	(S24)	(S24)	(S32)	(S32)	(S40)	(S40)
H	(S1)	(S1)	(S9)	(S9)	(S17)	(S17)	(S25)	(S25)	(S33)	(S33)	(S41)	(S41)

A-G = Standards

S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume is 210 μ l in all of the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- If the antioxidant level of the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.
- It is recommended that the samples and Trolox standards be assayed at least in duplicate (triplicate recommended).
- Monitor the absorbance at 750 nm or 405 nm using a plate reader.

Performing the Assay

1. Preparation of the Trolox standards: Take seven clean glass test tubes and mark them A-G. Add the amount of reconstituted Trolox and Assay Buffer to each tube as described in Table 1.

Tube	Reconstituted Trolox (μl)	Assay Buffer (μl)	Final Concentration (mM Trolox)
A	0	1,000	0
B	30	970	0.045
C	60	940	0.090
D	90	910	0.135
E	120	880	0.18
F	150	850	0.225
G	220	780	0.330

Table 1. Trolox standard preparation

2. **Trolox Standard Wells** - add 10 μl of Trolox standard (tubes A-G), 10 μl of Metmyoglobin, and 150 μl of Chromogen per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 10).
3. **Sample Wells** - add 10 μl of sample, 10 μl of Metmyoglobin, and 150 μl of Chromogen to two wells. To obtain reproducible results, antioxidant levels of the sample should fall within the standard curve. When necessary, samples can be diluted with Assay Buffer to bring antioxidants to this level.
4. Initiate the reactions by adding 40 μl of Hydrogen Peroxide Working Solution to all the wells being used. Add the Hydrogen Peroxide as quickly as possible (within one minute is recommended).
5. Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature. Remove the cover and read the absorbance at 750 nm or 405 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard and sample.
2. Plot the average absorbance of standards as a function of the final Trolox concentration (mM) from Table 1. A typical standard curve is shown below.

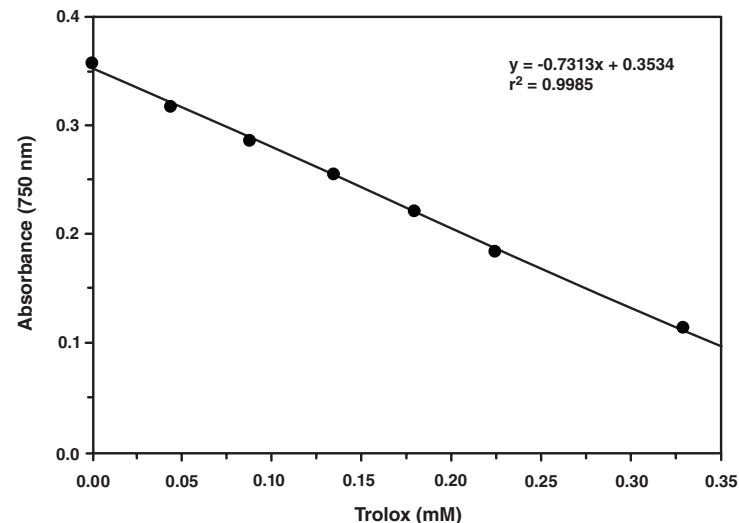


Figure 3. Trolox standard curve

3. Calculate the antioxidant concentration of the samples using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation.

$$\text{Antioxidant (mM)} = \frac{\text{Sample average absorbance} - (\text{y-intercept})}{\text{Slope}} \times \text{Dilution}$$

Performance Characteristics

Precision:

Inter-assay coefficient of variation = 3% (n = 20). Intra-assay coefficient of variation = 3.4% (n = 84).

Assay Range:

Samples containing antioxidants between 0.044-0.330 mM can be assayed without further dilution or concentration.

RESOURCES

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	Yes
	Borate	Yes
	HEPES (≤10 mM)	No
	Phosphate(≤10 mM)	No
Detergents:	Polysorbate 20 (≤1%)	No
	Triton X-100 (≤1%)	No
Protease Inhibitors/ Chelators:	Antipain (≤10 μg/ml)	No
	PMSF (≤200 μM)	No
	Leupeptin (≤10 μg/ml)	No
	Pepstatin (≤0.1 mg/ml)	No
	Chymostatin (≤5 μg/ml)	No
	EGTA (1 mM)	Yes
	EDTA (1 mM)	Yes
Others:	Glycerol (≤5%)	No
	Bovine serum albumin (≤0.1%)	No

Ethanol or dimethylsulfoxide (10 μl) can be used in the assay. However, the solvent (10 μl) needs to be added to the Trolox standard wells to compensate for the decrease in absorbance exerted by the solvent. Methanol interferes with the chromogen and can not be added to the assay.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No antioxidants were detected in the sample	Sample was too dilute	Re-assay the sample using a lower dilution
Absorbance <0.05 in the sample wells	The sample contains either a high concentration of antioxidants, or something that interferes with the assay	Dilute your sample with diluted Assay Buffer and re-assay
The Trolox standard curve did not work	Either the Trolox standards were not diluted properly or the Trolox standard has deteriorated	Reconstitute a new Trolox standard and set up the standards according to Table 1

Additional Reading

Go to www.caymanchem.com/709001/references for a list of publications citing the use of Cayman's Antioxidant Assay Kit.

References

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2. Miller, N., Rice-Evans, C., Davies, M.J., *et al.* A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **84**, 407-412 (1993).

3. Miller, N.J. and Rice-Evans, C. Factors influencing the antioxidant activity determined by the ABTS⁺ radical cation assay. *Free Rad. Res.* **26**, 195-199 (1997).
4. Miller, N.J., Rice-Evans, C., and Davies, M.J. A new method for measuring antioxidant activity. *Biochem. Soc. Trans.* **21**, 95S (1993).
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6. Benzie, I.F.F. and Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **239**, 70-76 (1996).
7. Kampa, M., Nistikaki, A., Tsaousis, V., *et al.* A new automated method for the determination of the total antioxidant capacity (TAC) of human plasma, based on the crocin bleaching assay. *BMC Clinical Pathology* **2**, 3-18 (2002).
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9. Koracevic, D., Koracevic, G., Djordjevic, V., *et al.* Method for the measurement of antioxidant activity in human fluids. *J. Clin. Pathol.* **54**, 356-361 (2001).

Related Products

- Ascorbate Assay Kit - Item No. 700420
- Aconitase Fluorometric Assay Kit - Item No. 700600
- Catalase Assay Kit - Item No. 707002
- Creatinine (urinary) Assay Kit - Item No. 500701
- Glutathione Assay Kit - Item No. 703002
- Glutathione Peroxide Assay Kit - Item No. 703102
- Glutathione Reductase Assay Kit - Item No. 703202
- Glutathione S-Transferase Assay Kit - Item No. 703302
- 8-hydroxy-2-deoxy Guanosine EIA Kit - Item No. 589320
- Hydrogen Peroxide Assay Kit - Item No. 706011

Related Products cont.

iPF_{2α}-VI EIA Kit - Item No. 516301
8-Isoprostane EIA Kit - Item No. 516351
Lipid Hydroperoxide Assay Kit - Item No. 705002
Protein Carbonyl Assay Kit - Item No. 10005020
Protein Carbonyl Fluorometric Assay Kit - Item No. 700490
Superoxide Dismutase Assay Kit - Item No. 706002
TBARS Assay Kit - Item No. 10009055
Thioredoxin Reductase Assay Kit - Item No. 10007892
Uric Acid Assay Kit - Item No. 700320
Xanthine Oxidase Assay Kit - Item No. 10010895

Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our **Warranty and Limitation of Remedy** located on our website and in our catalog.

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NOTES

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