Antioxidant Assay Kit

Item No. 709001

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
709002	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) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

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

Email: techserv@caymanchem.com

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 repeating 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 8). 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 (TE).

NOTE: Various matrices harbor a multitude of antioxidants, each present at distinct concentrations. Owing to the unique properties and reaction kinetics exhibited by individual antioxidant species within this assay scheme, sample linearity may not be observed. It is recommended that samples be processed and stored in the same manner prior to running the assay to obtain comparable Trolox equivalent antioxidant capacity (TEAC) values within the same experiment.

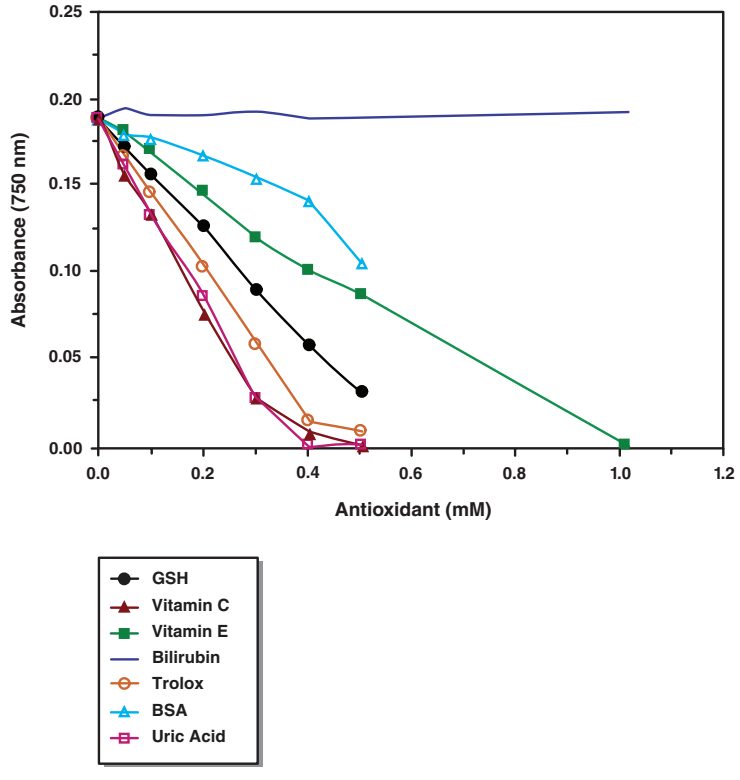


Figure 1. Inhibition of ABTS oxidation by antioxidants

PRE-ASSAY PREPARATION

Reagent Preparation

Some of the kit components are lyophilized or concentrated 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. 709002)**

   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) 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 concentration of this reconstituted reagent is 2.25 mM. It is used to prepare the Trolox standard curve (see p. 15). 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 µl of hydrogen peroxide with 990 µl of HPLC-grade water. Further dilute by removing 20 µl and diluting with 3.98 ml of HPLC-grade water to yield a 441 µM 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.

ASSAY PROTOCOL

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 22).

	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 µl 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	0.068
C	60	940	0.135
D	90	910	0.203
E	120	880	0.270
F	150	850	0.338
G	220	780	0.495

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 13).
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.

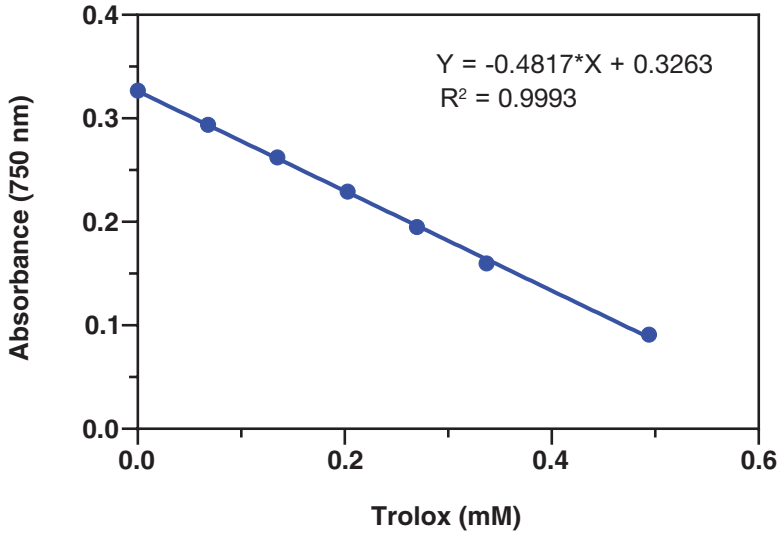


Figure 3. Trolox standard curve

3. Calculate the antioxidant capacity 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.

$$\text{Antioxidant Capacity (mM TE)} = \left[\frac{\text{(Sample average absorbance) - (y-intercept)}}{\text{Slope}}\right] \times \text{Dilution}$$

Performance Characteristics

Assay Range:

0.068 - 0.495 mM TE

Precision:

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

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

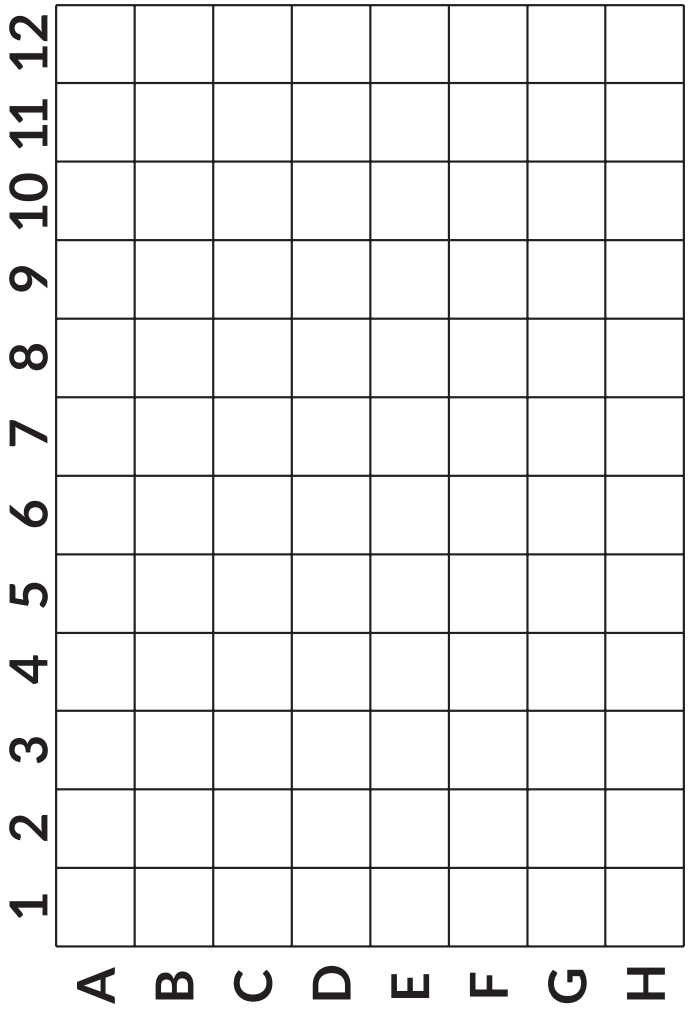
Ethanol or dimethylsulfoxide (10 µl) can be used in the assay. However, the solvent (10 µl) needs to be added to the Trolox standard wells to compensate for the decrease in absorbance exerted by the solvent. Methanol interferes with the chromogen and can not be added to the assay.

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	Reconstitiute a new Trolox standard and set up the standards according to Table 1

References

1. Halliwell, B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Rad. Res.* **25**, 57-74 (1996).
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 $ABTS^{\cdot+}$ 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).
5. Rice-Evans, C. and Miller, N. Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **234(24)**, 279-293 (1994).
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).
8. Miller, N.J., Johnston, J.D., Collis, C.S., *et al.* Serum total antioxidant activity after myocardial infarction. *Ann. Clin. Biochem.* **34**, 85-90 (1997).
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).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.