Activated ABTS Antioxidant Assay Kit

Cat# AOX-14

INSTRUCTION MANUAL ZBM0123.00

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier at 4°C.
All orders must be processed immediately upon arrival.

Trolox standard

Store at -20°C

Assay Buffer, ABTS, Activator, and Assay Plate

Store at room temperature

Long-term storage:

Remove the Trolox solution at -20°C. Reagents are good for at least 3 months after arrival if stored properly.

For *in vitro* Use Only

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress, however, excess ROS can overwhelm the systems and cause severe damage.

The Zen-Bio Activated ABTS Antioxidant Assay Kit can be used to determine the total antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. This assay improves upon the Zen-Bio AOX-1 kit by generating the highly colored $ABTS^{\bullet+}$ radical cation prior to testing samples for their antioxidant activity. This isolates a sample's ability to reduce the pre-formed $ABTS^{\bullet+}$ radical cation from also interacting with ferryl metmyoglobin or $HO^{\bullet}$ which can overestimate its antioxidant activity. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control reducing the radical cation in a dose dependent manner. The antioxidant activity in biological fluids, cells, tissues, and natural extracts can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present. This assay measures radical scavenging by electron donation and when combined with Zen-Bio's other antioxidant assay kits, provides a comprehensive analysis of a test sample's antioxidant activity.

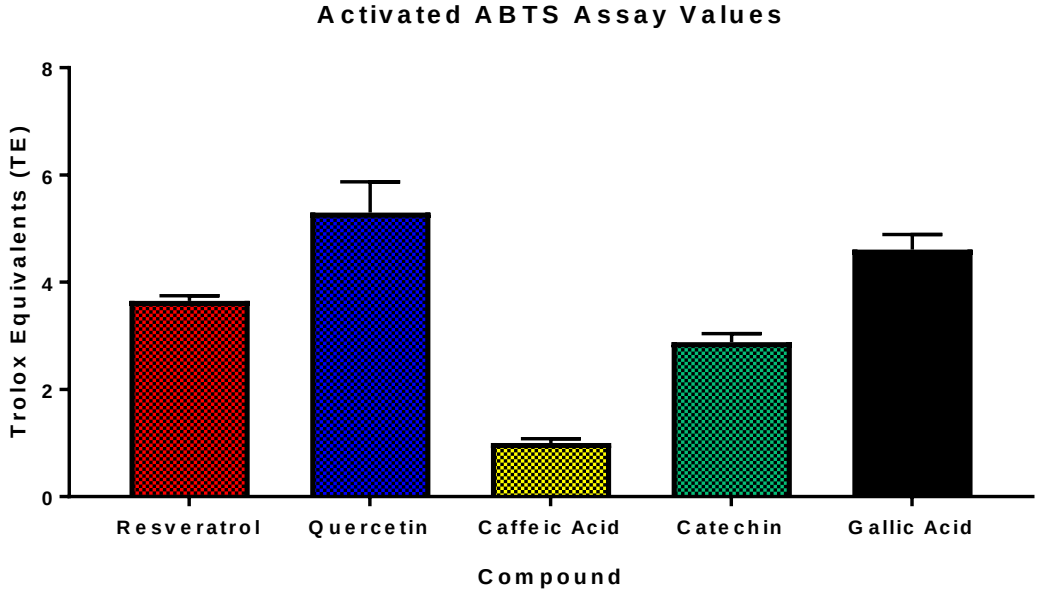


Figure 1. Effects of Antioxidants in the Activated ABTS Assay

Resveratrol, Quercetin, Caffeic Acid, Catechin, and Gallic acid were tested for their antioxidant activity in the Activated ABTS antioxidant assay as compared to Trolox.

PRINCIPLE OF THE ASSAY

The Activator included in this kit can oxidize ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, $ABTS^{\bullet+}$, that is green in color and can be measured by absorbance at 405nm. Antioxidants can reduce the ABTS radical cation back to ABTS by electron donation radical scavenging. The concentration of antioxidant in the test sample is inversely proportional to the $ABTS^{\bullet+}$ 405nm absorbance.

ABTS + Activator ⟶ $ABTS^{\bullet+}$ (highly colored)

$ABTS^{\bullet+}$ + Antioxidant ⟶ ABTS (less color)

[Antioxidants reduce the ABTS radical cation by electron transfer radical scavenging]

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Color	UNIT	QTY	STORAGE
Blank Assay Plates	96-well assay plates, blank	---	PLATE	1	-----
AOX Assay Buffer	50 ml	CLEAR	BOTTLE	1	RT
ABTS powder	10 mg	AMBER	VIAL	1	RT
Activator powder	100 mg	CLEAR	BOTTLE	1	RT
AOX Trolox	5mM in Dilution Buffer	AMBER	50 µl /VIAL	1	-20°C
Tray	For multi-channel pipetters, clear polyvinyl	---	EACH	2	RT

Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Distilled water
- Plate reader with a filter of 405 nm

Reagents that might interfere with the assay results:

TWEEN 20	2-mercaptoethanol	EDTA
> 0.1% TRITON X-100	> 500mM Tris	> 5% DMSO
IGEPAL CA-630 (Nonidet P-40)	SDS	100mM HEPES
> 0.2% CHAPS	DTT	NP-40

SAMPLE PREPARATION

Cell Lysate Preparation

1. Scrape ~1 x10^6 cells and centrifuge at 1,000xg to prepare a cell pellet. DO NOT use proteolytic enzymes such as trypsin but scrape using a rubber policeman or cell scraper tool.
2. Homogenize or sonicate the cell pellet on ice in 1ml cold PBS or AOX Assay buffer
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and keep on ice until ready to use in the assay.
5. If not using the same day, store the samples at -80°C.
6. Data is expressed as Trolox equivalents (TE) per cell number (i.e. µmole TE/10^6 cells)

Tissue Lysate Preparation

1. Homogenize tissue samples on ice in cold buffer at ~10-20mg tissue per ml cold PBS or buffer
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

Plasma Preparation

1. Collect the blood in a tube containing heparin (preferable) or other anticoagulant.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

Serum Preparation

1. Collect the blood in a tube WITHOUT any anticoagulant. Allow the blood to clot.
2. Centrifuge at 2,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

Saliva Collection

1. Collect whole saliva for a defined period of time (i.e. 1-5 minutes) into polypropylene tubes.
2. Immediately place on ice or store at -80°C for later analysis.
3. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

Food Extract Preparation

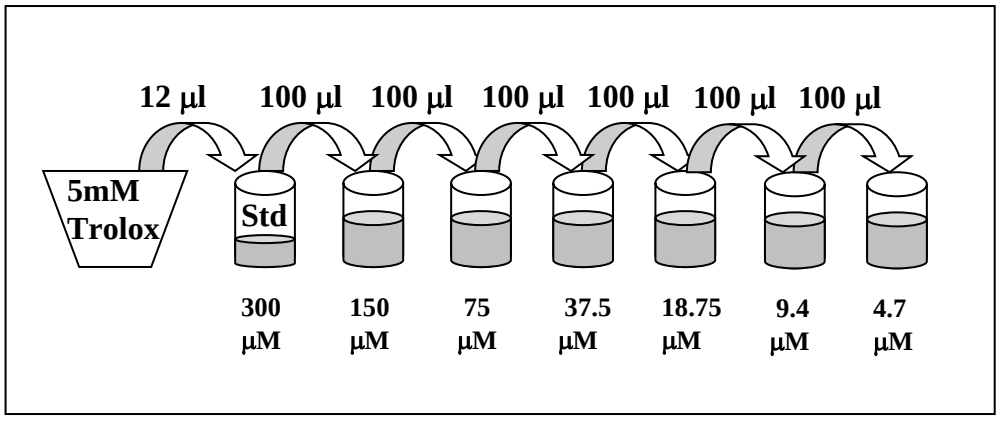
1. Weigh the starting material.
2. Homogenize in a small volume ice cold buffer or water.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

ASSAY PROCEDURE

IMPORTANT NOTE: ABTS solution must be activated for at least 4 hours prior to assay. Once activated, the ABTS solution can be stored at 4°C for up to 48 hours.

1. Prepare the Activator Solution by weighing out 10mg of Activator and dissolving in 1.5mL distilled water. This creates a 10x stock of Activator solution. Prepare 2.0 mL of 1x Activator Solution by diluting 200µl 10x stock into 1.8mL of distilled water. The remaining 10x stock can be stored at 4°C for 24 hours.
2. Weigh out 5mg of ABTS powder and dissolve in 1.3mL of 1x Activator Solution. Mix and incubate in the dark at room temperature for at least 4 hours. After 4 hours the Activated ABTS solution can be used immediately or stored at 4°C for 48 hours.
3. Prepare Trolox standards in a 96-well plate for easy transfer as follows:

   Briefly spin down the contents of the 5 mM Trolox standard tube after thawing. Pipette 12 µL of the 5 mM Trolox standard into 188 µL Assay Buffer. This produces the top Trolox standard of 300 µM. Pipette 100 µl of assay buffer into 6 wells of a 96-well plate. Using the newly diluted 300 µM Trolox solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 300 µM stock dilution serves as the highest standard, and the assay buffer serves as the zero standard.

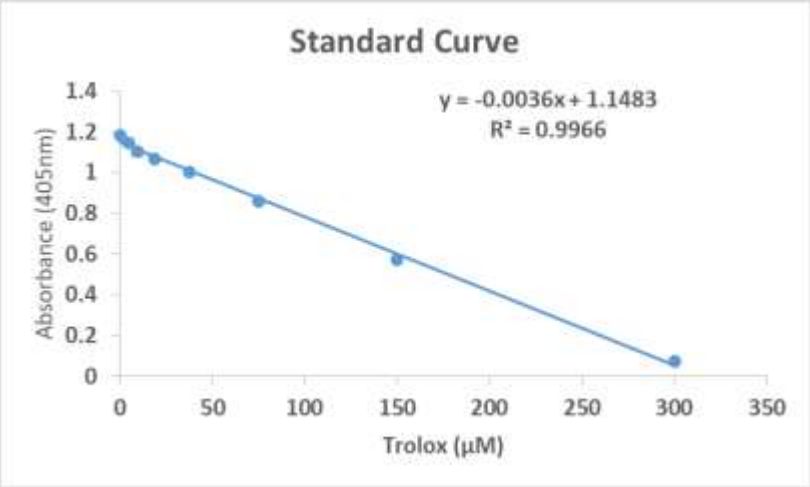


4. Prepare test sample dilutions as necessary.
5. Add 25 µL of samples or Trolox standards to individual wells of the assay plate provided, add 25 µL of assay buffer to individual wells as a negative control (0 standard).
6. Dilute the Activated ABTS Solution 50-fold by adding 300 µL to 14.7mL of Assay Buffer. Mix thoroughly.
7. To begin the assay, add 150 µl of the Diluted Activated ABTS solution per well and place on plate shaker at room temperature. Allow the reaction to proceed for 5 minutes protected from light.
8. Immediately read absorbance using plate reader at a wavelength of 405 nm.

TROLOX STANDARD CURVE

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

µM Trolox	OD	stdev
0	0.0756	0.013
4.7	0.5719	0.007
9.4	0.8578	0.003
18.75	1.0058	0.016
37.5	1.0693	0.009
75	1.1041	0.021
150	1.1469	0.010
300	1.1853	0.013



slope =	-0.0036
intercept=	1.1483
R^2=	0.9966

y = observed O.D.
x = concentration of Trolox in µM
To calculate x for each y, (i.e. to change the observed O.D. into Trolox equivalent concentration) use the following equation:
y= (slope) times (x) plus intercept

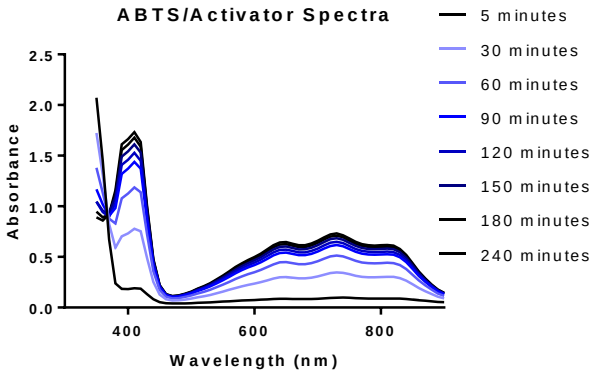
y= mx+b so x=(y-b)/m

x= (y – 1.1483)/(-0.0036) where (-0.0036)= slope of the line and1.1483= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

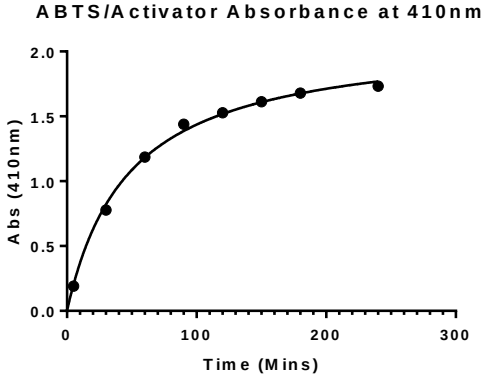
The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Antioxidant activity is expressed as µM Trolox equivalents (TE) or µmol Trolox/g sample.

ABTS ACTIVATION KINETICS



Absorbance spectra changes with time



410nm absorbance plateaus at 4 hours

APPENDIX A: Plate layout _____

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX B: Protocol Flowchart

ACTIVATED ABTS ASSAY

Prior to assay, activate the ABTS using 1x Activator solution and allow to incubate for **at least 4 hours** at room temperature.

↓

Make necessary test compound dilutions in Assay Buffer.

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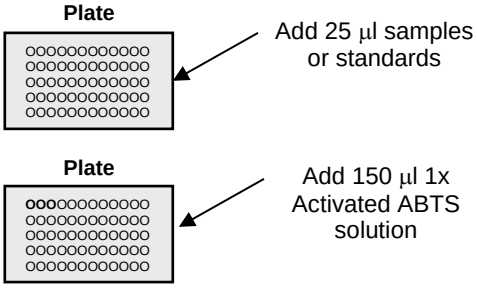
Add 25 µl/well samples or standards to blank assay plate.



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Start the assay by adding 150 µl/well **1x** Activated ABTS solution.

↓

Incubate at 25˚C (room temperature) for 5 minutes.

↓

Measure the optical density of each well at 405 nm using a spectrophotometer plate reader.

REFERENCES

1. Clin. Sci., **84**:407–412, 1993.
2. Free Rad. Bio. & Med., **26**(9/10):1231-1237, 1999

FREQUENTLY ASKED QUESTIONS _____

1. **Is it alright that my absorbance values are lower than those in the sample data but still generate a good Trolox standard curve?** Yes, the absorbance values may vary from different plate readers, however the zero standard should give an absorbance value near 1. If the Trolox standards still generate a robust standard curve, the assay is functioning properly.

2. **Should I dilute my sample for testing its AOX activity**? In order to accurately determine the AOX activity of your sample, the absorbance value must fall on the Trolox standard curve. We recommend preparing several serial dilutions of your test sample and using the AOX assay buffer to ensure that you generate usable absorbance values. Preferably, your sample absorbance values should be in the middle of the standard curve to ensure the most accurate reading.

3. **Can this assay be used for lipophilic samples?** This assay can be modified to support measuring AOX values of lipohilic samples in ethanol. Prepare the undiluted ABTS/Activator solution as described in this protocol. Prepare the Trolox standards using 100% ethanol instead of assay buffer. Dilute the ABTS/Activator solution 50-fold using 75% ethanol instead of assay buffer. Proceed with the assay as described.