ABTS Antioxidant Assay Kit

Cat# AOX-1

INSTRUCTION MANUAL ZBM0034.03

STORAGE CONDITIONS

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

ABTS Solution

Store at 4°C.

Trolox standard and Myoglobin reagent

Store at -20°C

Assay Buffer, Dilution Buffer, Stop Solution, and Assay Plate

Store at room temperature

Long-term storage:

Remove the ABTS solution from the box and place at 4°C, store the Trolox and Myoglobin solutions 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 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. The assay measures $ABTS^{\cdot+}$ radical cation formation induced by metmyoglobin and hydrogen peroxide. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting the formation of 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 ORAC antioxidant assay kit, provides a comprehensive analysis of a test sample's antioxidant activity.

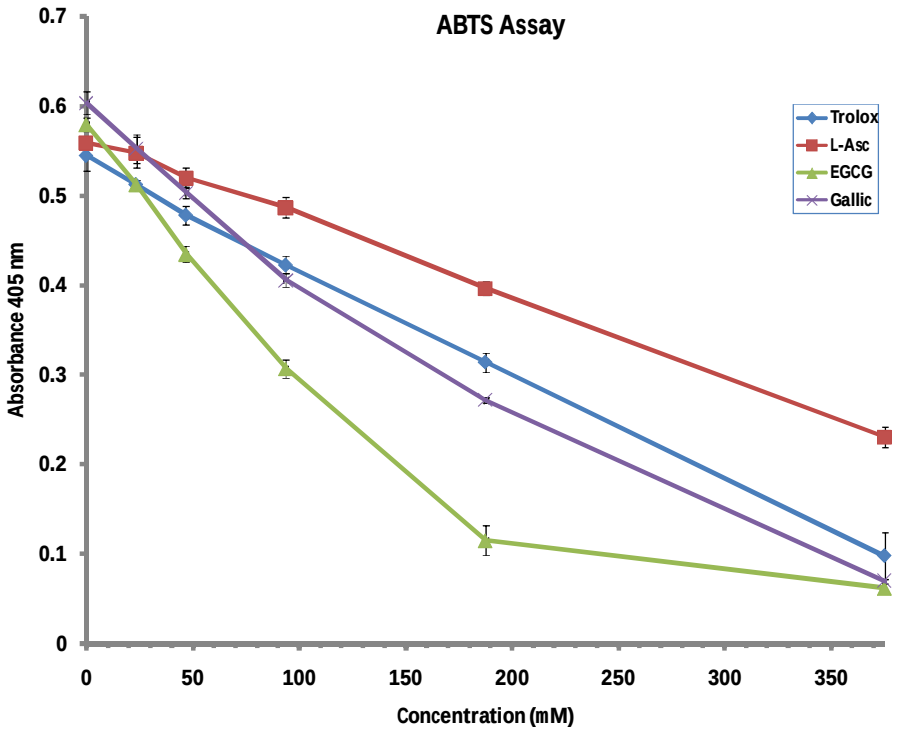
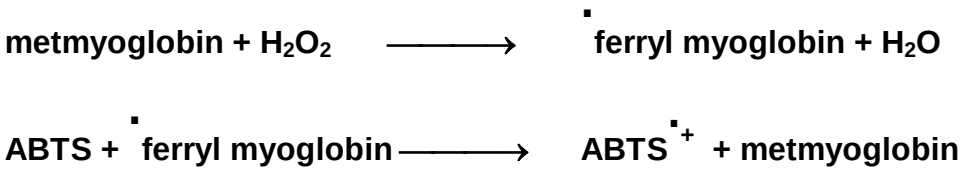


Figure 1. Effects of antioxidants in ABTS assay

Trolox, Sodium L-ascorbate (L-Asc), Epigallocatechin gallate (EGCG), and Gallic acid (Gallic) were tested for their antioxidant activity in the ABTS antioxidant assay.

PRINCIPLE OF THE ASSAY

A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, $ABTS^{\cdot+}$, that is green in color and can be measured by absorbance at 405nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical. The concentration of antioxidant in the test sample is inversely proportional to the ABTS radical formation and 405nm absorbance.



[Antioxidants inhibit the oxidation of ABTS 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 Dilution Buffer	2.475 ml	AMBER	BOTTLE	1	RT
AOX Assay Buffer	11 ml	CLEAR	BOTTLE	1	RT
ABTS solution	11 ml	CLEAR	BOTTLE	1	4°C
Stop Solution	6ml	CLEAR	BOTTLE	1	RT
AOX Trolox	1.5mM in Dilution Buffer	AMBER	20 µl /VIAL	1	-20°C
Myoglobin Solution	100x stock	AMBER	30 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
- Plate reader with a filter of 405 nm

Reagents that might interfere with the assay results:

> 1% TWEEN 20	2-mercaptoethanol
> 1% TRITON X-100	Tris
IGEPAL CA-630 (Nonidet P-40)	Borate
> 0.2% CHAPS	DTT

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 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 ~200mg tissue per ml cold 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 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

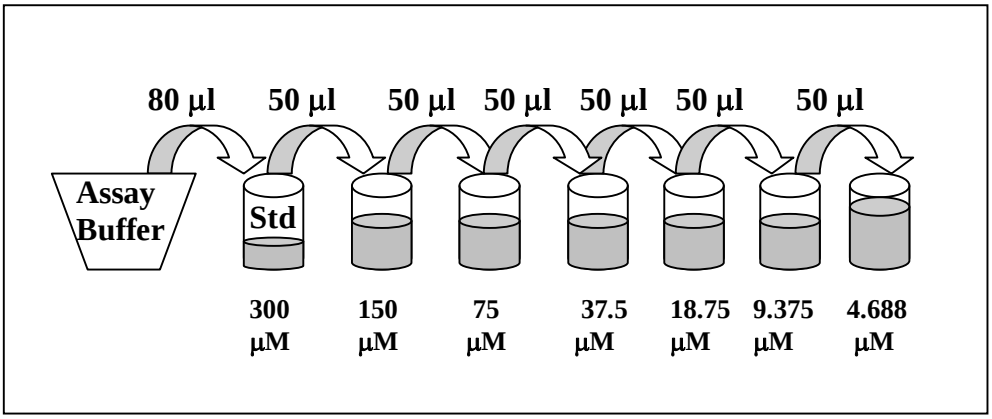
1. Weigh the starting material.
2. Homogenize in a small volume ice cold buffer or water.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

ASSAY PROCEDURE

1. Remove the ABTS solution from the refrigerator and allow it to come to room temperature.

2. Prepare Trolox standards as follows:

   Briefly spin down the contents of the 1.5 mM Trolox standard tube after thawing. Pipette 80 μl of Assay Buffer into the 1.5 mM Trolox standard tube provided and mix well by vortexing. This produces a diluted stock Trolox standard of 300 μM. Pipette 50 μl of assay buffer into 6 tubes (not provided). Using the newly diluted stock 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.



3. Prepare the Myoglobin Working Solution. Briefly spin down the contents of the Myoglobin stock solution tube. Add 25μl Myoglobin stock solution to the Dilution Buffer bottle (contains 2.475 ml) and gently invert. Place the working solution on ice until needed.

4. Add 10 μl of samples or Trolox standards to individual wells of the assay plate provided, add 10 μl of assay buffer to individual wells as a negative control.

5. Add 20 μl of the myoglobin working solution to each of the wells containing standards and samples from step 4.

6. To begin the assay, add 100 μl of the ABTS solution per well and place on plate shaker at room temperature. Allow the reaction to proceed for 5 minutes. To stop the reaction, add 50 μl of Stop Solution per well.

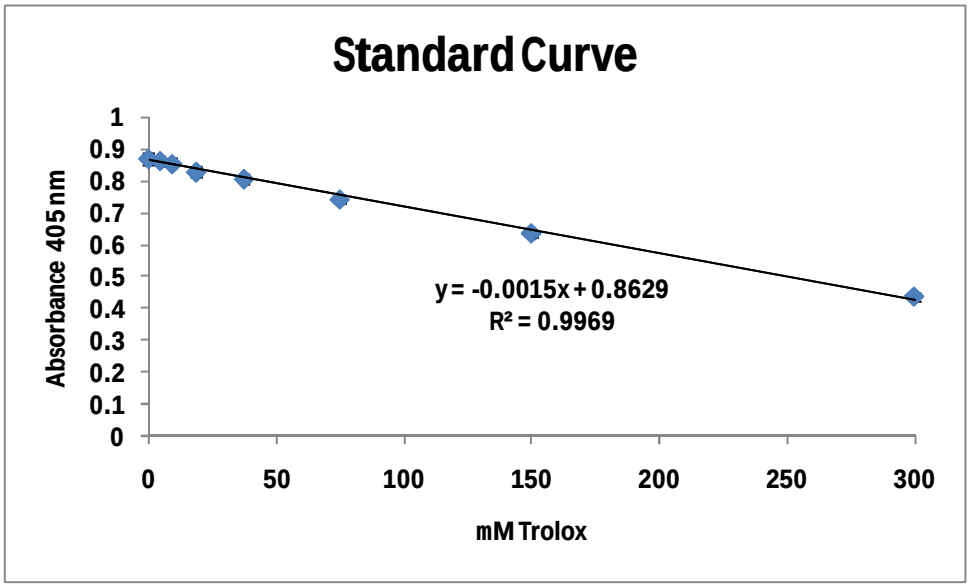
7. Read absorbance using plate reader at a wavelength of 405 nm.

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	0.871	0.019
4.6875	0.864	0.006
9.375	0.855	0.003
18.75	0.829	0.018
37.5	0.806	0.006
75	0.742	0.013
150	0.636	0.009
300	0.436	0.013

slope =	-0.0015
intercept=	0.8629
R^2=	0.9969



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 – 0.8629)/(-.0015) where (-.0015)= slope of the line and 0.8629= 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).

OPTIONAL KINETIC ASSAY PROCEDURE

1. Prepare Trolox and myoglobin reagents as described above.

2. Set-up plate reader for kinetic reading mode:
   Total time = 10 minutes
   Interval = 30 seconds
   Absorbance= 405nm

3. Add 10 μl of samples or Trolox standards to individual wells of the assay plate provided, add 10 μl of assay buffer to individual wells as a negative control.

4. Add 20 μl of the myoglobin working solution to each of the wells containing standards and samples from step 3.

5. To begin the assay, add 100 μl of the ABTS solution per well and place in plate reader and begin reading absorbance using the kinetic mode.

TROLOX KINETIC STANDARD CURVE

Trolox concentration (μM)

Time (min.)	1500	750	375	187.5	93.75	46.875	23.4375	0
0	0.059	0.058667	0.060333	0.062	0.059667	0.080333	0.109333	0.142
0.5	0.055667	0.056	0.058	0.059333	0.102667	0.159333	0.189667	0.219333
1	0.055667	0.056333	0.057333	0.076333	0.183333	0.238667	0.268667	0.3
1.5	0.055667	0.055667	0.057667	0.154667	0.262	0.318333	0.35	0.381333
2	0.055333	0.055667	0.058	0.233667	0.341667	0.398	0.431333	0.463
2.5	0.056	0.056333	0.098333	0.314333	0.422667	0.478333	0.512667	0.545
3	0.056333	0.056667	0.175667	0.394667	0.503	0.558	0.592667	0.625333
3.5	0.056	0.056333	0.252	0.474	0.582	0.636667	0.672	0.704
4	0.056667	0.056667	0.328667	0.554333	0.662	0.715333	0.751333	0.783
4.5	0.057	0.057	0.405667	0.634667	0.742	0.794	0.830667	0.862333
5	0.056333	0.056667	0.483	0.715333	0.820667	0.871333	0.909333	0.941333
5.5	0.056667	0.077667	0.563333	0.797667	0.900667	0.95	0.99	1.024
6	0.057333	0.154333	0.645	0.881333	0.981	1.032333	1.072	1.109
6.5	0.057333	0.233667	0.729	0.966667	1.063	1.116333	1.155667	1.196
7	0.057	0.313333	0.813	1.053	1.145333	1.201667	1.240667	1.283
7.5	0.057333	0.396667	0.899333	1.142333	1.230667	1.29	1.328	1.372333
8	0.058	0.481667	0.987667	1.233333	1.319333	1.380333	1.417667	1.462667
8.5	0.058333	0.567667	1.075667	1.325667	1.408667	1.470333	1.507333	1.553
9	0.058333	0.654667	1.164667	1.419	1.500333	1.562667	1.599	1.644
9.5	0.059	0.743	1.254667	1.512667	1.594333	1.656333	1.692	1.736333
10	0.059333	0.832333	1.346667	1.608	1.690667	1.751333	1.785667	1.829333

	AUC
1500	1.668667
750	5.160833
375	11.34033
187.5	15.309
93.75	17.17033
46.875	18.284
23.4375	19.01283
0	19.76167

Area under the curve (AUC)
AUC= 0.5+ f0+f1+...+f20+(0.5*f21)
Where fx = Abs value at time points 0 – 10 min

Figure 2. Absorbance over time for Trolox standards

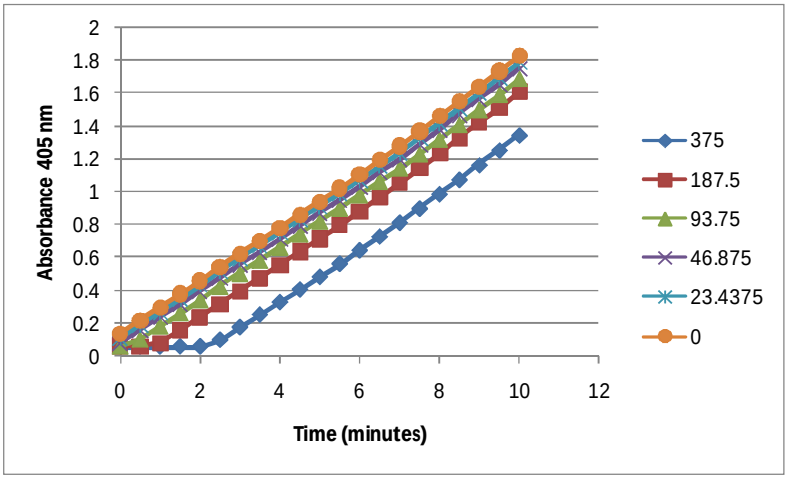
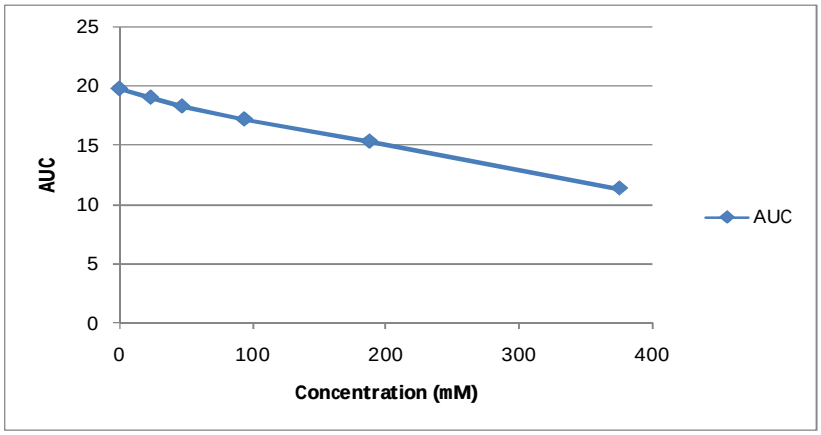


Figure 3. AUC values for increasing Trolox concentrations



Comparison of Stopped and Kinetic Assays

Figure 4. ABTS Absorbance Values at 5 Minutes

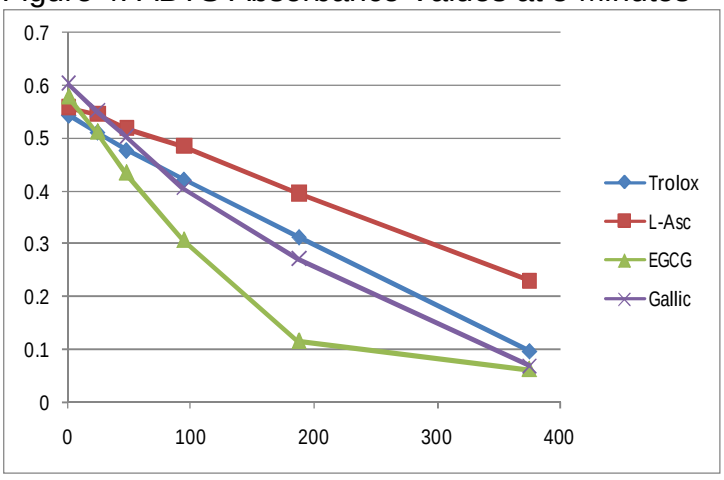
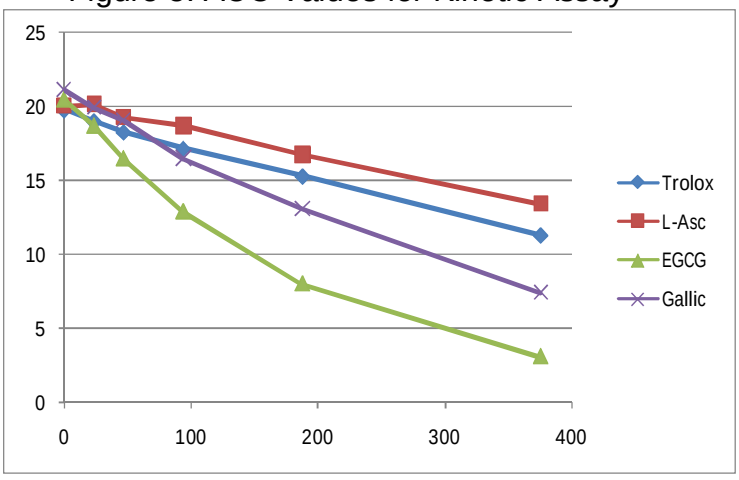


Figure 5. AUC Values for Kinetic Assay



Trolox =Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid]
L-Asc = Sodium L-ascorbate
EGCG = Epigallocatechin gallate
Gallic= Gallic acid

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

ABTS ASSAY

Make necessary test compound dilutions in Assay Buffer.

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Prior to assay, allow ABTS solution to equilibrate to room temperature, prepare myoglobin dilution and standards. Keep Trolox and myoglobin solutions on ice until used.

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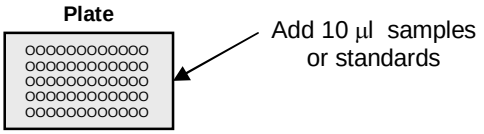
Add 10 µl/well samples or standards to blank assay plate.



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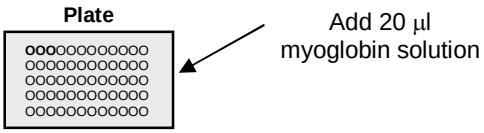
Add 20 µl/well myoglobin dilution to the plate (including the Trolox standards).



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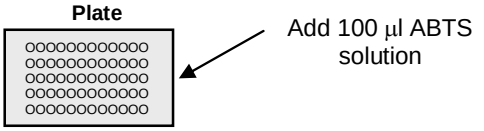
Start assay by adding 100 µl/well ABTS solution.



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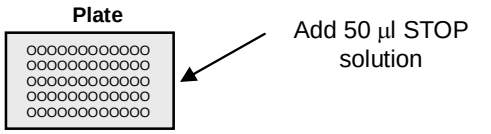
Incubate at 25°C (room temperature) for 5 minutes and stop reaction by adding 50 µl/well stop solution.



Optionally, perform kinetic assay by placing in plate reader and measure absorbance at 405 nm every 30 seconds for 10 minutes.

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

REFERENCES

1. Clin. Chem., 44(6):1309–1315, 1998.
2. J. Clin. Biochem. Nutr., 44: 46–51, January 2009.
3. Indian J Biochem & Biophy. 46, 126-129 February 2009.