ORAC Antioxidant Assay Kit
(60 point kit)
Cat# AOX-2

INSTRUCTION MANUAL ZBM0035.03

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier at 4°C.
All orders must be processed immediately upon arrival. Any adverse conditions upon arrival must be reported within 7 days.

Fluorescein Solution
Remove from box and store at 4°C
Trolox Standard and AAPH Reagent
Remove from box and store at -20°C
AOX Assay Buffer and black assay plate
Store at Room Temperature
Long-term Storage
If stored properly, the reagents are good until the expiration date listed on the kit.

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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TABLE OF CONTENTS

	PAGE#
Introduction	3
Principle of Assay	4
Items Included in the Kit	4
Sample Preparation	5
Assay Procedure	6
Trolox Standard Curve	7
Appendix A: Plate layout	8
Appendix B: Protocol Flowchart	9
References	9

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 ORAC (Oxygen Radical Absorbance Capacity) 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 the loss of fluorescein fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride). Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting fluorescein decay in a dose dependent manner. The ORAC assay is a kinetic assay measuring fluroescein decay and antioxidant protection over time. 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 antioxidant activity by hydrogen atom transfer and when combined with Zen-Bio's ABTS antioxidant assay kit, provides a comprehensive analysis of a test sample's antioxidant activity.

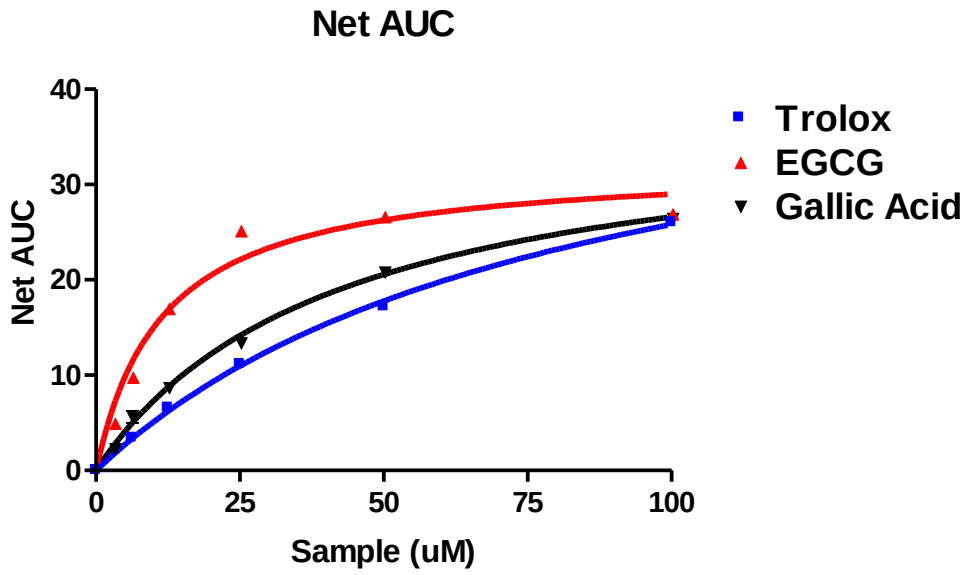
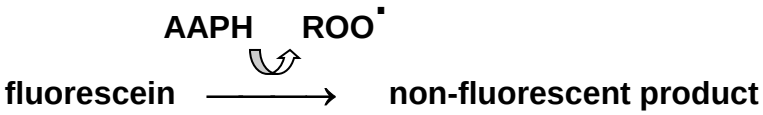


Figure 1. Effects of antioxidants in ORAC assay

Trolox, Epigallocatechin gallate (EGCG), and Gallic acid were tested for their antioxidant activity in the ORAC antioxidant assay.

PRINCIPLE OF THE ASSAY

A peroxyl radical (ROO•) is formed from the breakdown of AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) at 37 °C. The peroxyl radical can oxidize fluorescein (3',6'-dihydroxy-spiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) to generate a product without fluorescence. Antioxidants suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured over 30 minutes by excitation at 485 nm, emission at 538 nm, and cutoff=530 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a known antioxidant, trolox.



[Antioxidants inhibit the oxidation of fluorescein by hydrogen atom transfer]

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Blank Assay Plates	96-**well assay plates, black clear bottom**	---	PLATE	1	-----
AOX Assay Buffer	50 ml	---	BOTTLE	1	RT
AAPH	**130 mg**	---	BOTTLE	1	-20°C
Trolox (AOX-2)	1.5mM in AOX Buffer		20 µl /VIAL	1	-20°C
Fluorescein Solution	**60x stock**		**300 µl /VIAL**	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl	---	EACH	1	-----

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

- Multi-channel Pipet , single channel pipet and pipet tips
- Tubes for preparing standards and working solutions
- Fluorescence plate reader able to perform excitation=485nm; emission=528 -538nm (cutoff=530nm, if necessary)
- Fluorescence plate reader with incubator chamber set to 37°C

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) **[Dilute 100-fold in assay buffer prior to assaying].**

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) **[Dilute 100-fold in assay buffer prior to assaying].**

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

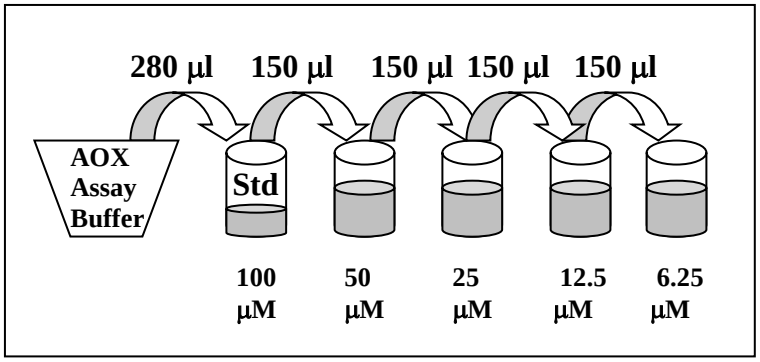
1. Weigh the starting material.
2. Homogenize in a small volume ice cold buffer or water.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

ASSAY PROCEDURE

THIS KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 60 WELLS. AT LEAST 6 OF THESE WELLS ARE REQUIRED FOR TROLOX STANDARDS

1. Equilibrate the plate reader incubation chamber to 37°C before beginning. Set-up plate reader to perform a kinetic read for 30 minutes with 1 minute intervals. Excitation = 485 nm; Emission = 528 - 538 nm (Cutoff = 530 nm, if required). **SET PLATE READER TO BOTTOM READ.**
2. Prepare fluorescein working solution from the stock solution provided by transferring **11.8ml** of AOX Assay Buffer to an empty tube (not provided) and adding **0.2ml** stock fluorescein solution. Mix and protect from light.
3. Prepare Trolox standards as follows:

   Briefly spin down the contents of the 1.5 mM Trolox standard tube after thawing. Pipette **280 µl** of AOX Assay Buffer into the 1.5 mM Trolox standard tube provided and mix well by vortexing. This produces a diluted stock Trolox standard of **100 µM**. Pipette **150 µl** of AOX Assay Buffer into 4 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 **100 µM** stock dilution serves as the highest standard, and the assay buffer serves as the zero standard.



4. Add 150 µl of the working fluorescein solution to each of the **INNER 60 WELLS** of the assay plate provided.
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. Place plate at 37°C for at least **10** minutes. [**IF THE AOX ACTIVITY OF THE TEST SAMPLES IS UNKNOWN, WE RECOMMEND PREPARING SEVERAL DILUTIONS IN AOX ASSAY BUFFER.]**
6. While the assay plate is equilibrating to 37°C, prepare the AAPH Working Solution by adding 2.0 ml AOX Assay Buffer to the AAPH tube provided and gently invert. Place the working solution on ice until needed. AAPH solution is good for 8 hours if kept on ice.
7. To begin the assay, add 25 µl of the AAPH working solution to each of the wells containing standards and samples from step 5. Place the assay plate in the plate reader and begin kinetic fluorescence reading.

TROLOX STANDARD CURVE

Generate standard curve: see example below (Collected using BioTek Synergy 2)
[DO NOT use this standard curve to generate your data. This is an example.]

Kinetic RLU Values

	Concentration of Trolox in uM					
Time	100	50	25	12.5	6.25	0
0	55992	56344	56351	56384	56956	55535
1	56216	56460	56586	56454	56896	48138
2	56037	56398	56519	56397	55631	40247
3	55967	56275	56583	55338	52547	32741
4	56137	56382	56417	52187	48307	26342
5	56002	56107	56406	48270	44237	20703
6	56036	56238	56089	44984	40712	15902
7	56007	56119	54181	42499	38192	11780
8	56085	56119	51297	40379	35385	8398
9	55932	56115	48083	38903	30843	5742
10	55942	55336	45209	37387	24642	3685
11	56021	52884	42861	35772	18978	2382
12	55923	50053	41026	34089	13808	1593
13	55753	47606	39195	30718	9707	1156
14	55347	45531	37712	24423	6410	974
15	53792	43830	35980	18277	4080	875
16	51709	42308	32518	13004	2535	819
17	49619	40913	25201	8784	1646	794
18	48051	40004	18260	5633	1166	795
19	46846	38642	12330	3501	946	796
20	45810	37242	7873	2187	864	798
21	44909	33638	4745	1440	810	799
22	44282	23825	2878	1088	832	775
23	43620	15079	1738	910	799	784
24	43243	8892	1223	832	793	791
25	42812	4828	974	820	786	770
26	42766	2581	859	809	779	769
27	42359	1490	830	803	782	786
28	42083	1064	826	791	764	778
29	41648	901	807	787	814	782
30	41021	821	803	799	787	787

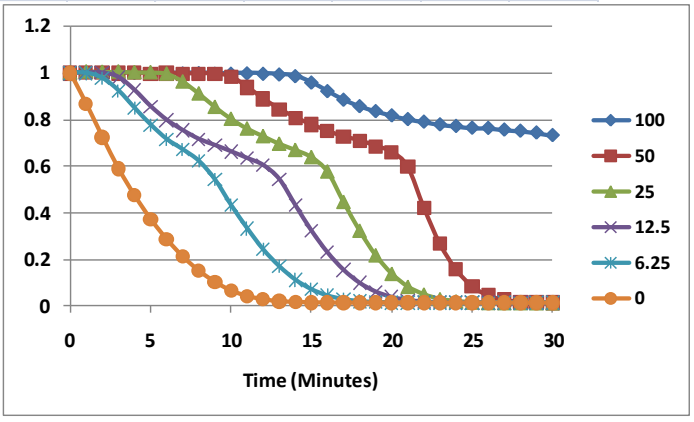
Normalized to Time=0 by (RLU/RLU0)

	Concentration of Trolox in uM					
Time	100	50	25	12.5	6.25	0
0	1	1	1	1	1	1
1	1.004001	1.002059	1.00417	1.001241	0.998947	0.866805
2	1.000804	1.000958	1.002981	1.000231	0.976736	0.724714
3	0.999554	0.998775	1.004117	0.981449	0.922589	0.589556
4	1.00259	1.000674	1.001171	0.925564	0.848146	0.474332
5	1.000179	0.995794	1.000976	0.856094	0.776687	0.372792
6	1.000786	0.998119	0.995351	0.797815	0.714797	0.286342
7	1.000268	0.996007	0.961491	0.753742	0.670553	0.212118
8	1.001661	0.996007	0.910312	0.716143	0.621269	0.15122
9	0.998928	0.995936	0.853277	0.689965	0.541523	0.103394
10	0.999107	0.98211	0.802275	0.663078	0.43265	0.066355
11	1.000518	0.938592	0.760608	0.634435	0.333205	0.042892
12	0.998768	0.888347	0.728044	0.604586	0.242433	0.028685
13	0.995732	0.844917	0.695551	0.5448	0.17043	0.020816
14	0.98848	0.80809	0.669234	0.433155	0.112543	0.017538
15	0.960709	0.7779	0.638498	0.324152	0.071634	0.015756
16	0.923507	0.750887	0.577062	0.230633	0.044508	0.014747
17	0.88618	0.726129	0.447215	0.155789	0.0289	0.014297
18	0.858176	0.709996	0.32404	0.099904	0.020472	0.014315
19	0.836655	0.685823	0.218807	0.062092	0.016609	0.014333
20	0.818153	0.660975	0.139714	0.038788	0.01517	0.014369
21	0.802061	0.597011	0.084204	0.025539	0.014222	0.014387
22	0.790863	0.422849	0.051073	0.019296	0.014608	0.013955
23	0.77904	0.267624	0.030842	0.016139	0.014028	0.014117
24	0.772307	0.157816	0.021703	0.014756	0.013923	0.014243
25	0.764609	0.085688	0.017285	0.014543	0.0138	0.013865
26	0.763788	0.045808	0.015244	0.014348	0.013677	0.013847
27	0.756519	0.026445	0.014729	0.014242	0.01373	0.014153
28	0.75159	0.018884	0.014658	0.014029	0.013414	0.014009
29	0.743821	0.015991	0.014321	0.013958	0.014292	0.014081
30	0.732623	0.014571	0.01425	0.014171	0.013818	0.014171

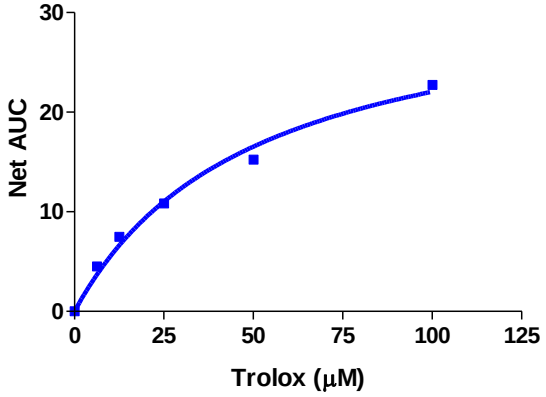
Use normalized data to generate Area Under the Curve (AUC) values. AUC values can be calculated by a statistical program (such as GraphPad Prism) or by the following formula:

AUC= 0.5 + (F1/F0) + (F2/F0) + …+ 0.5*(F30/F0)
Where F0= normalized fluorescence at t=0

Net AUC is determined by subtracting the AUC for no compound addition from the other AUC values.



	100	50	25	12.5	6.125	0
AUC	27.9	20.4	16.0	12.6	9.6	5.1
Net AUC	22.7	15.2	10.8	7.4	4.5	0



Data for unknowns may be expressed as µM Trolox equivalents or µmole Trolox/gram.

APPENDIX A: Plate layout_____

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

APPENDIX B: Protocol Flowchart

ORAC ASSAY

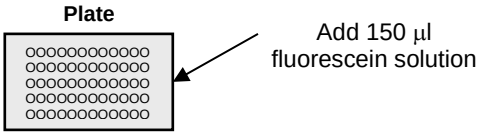
Make necessary test compound dilutions in Assay Buffer.

↓

Prior to assay, warm plate chamber to 37°C, prepare fluorescein working solution and trolox standards.

↓

Add 150 µl/well Working Fluorescein Solution to blank assay plate.



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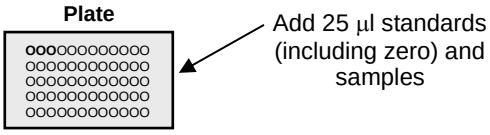
Add 25 µl/well trolox standards and test samples to fluorescein containing wells and place in incubator at 37°C for 10 minutes.



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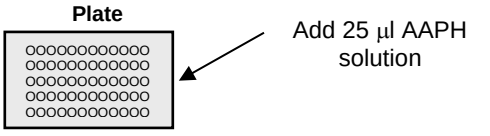
Prepare AAPH solution. Add 25 µl/well of AAPH working solution and place assay plate in plate reader. Begin kinetic fluorescence read. Excitation= 485 nm; Emission=528 - 538 nm; (Cutoff=530 nm, if necessary)



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3. J. Clin. Biochem. Nutr., 44: 46–51, Jan 2009.
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5. Free Radical Biol Med. 14(3):303-311, 1993.
6. J Agr and Food Chem. 49:4619-4626, 2001.

FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my fluorescence values are lower than those in the sample data but still generate a good Trolox standard curve?** Yes, the relative fluorescence values detected by the fluorimeter are based on the sensitivity of the instrument used. Our data was collected using a BioTek Synergy II fluorimeter, other instruments vary in sensitivity and can give lower values. If the Trolox standards still generate a robust standard curve, the assay is functioning appropriately.

2. **Should I dilute my sample for testing its AOX activity?** In order to accurately determine the AOX activity of your sample, the Net AUC value must fall on the Trolox Net AUC standard curve. We recommend preparing several serial dilutions of your test sample using the AOX assay buffer to ensure that you generate usable Net AUC values.