ORAC Antioxidant Assay Kit

Cat# AOX-2-RB (300 point kit)

INSTRUCTION MANUAL ZBM0059.06

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority service or other priority service 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 plates
Store at Room Temperature

Long-term Storage
The AOX-2 reagents are suitable for use for at least 3 months upon arrival if stored properly.

FOR *IN VITRO* RESEARCH USES ONLY. NOT TO BE USED FOR DIAGNOSTIC OR THERAPEUTIC PURPOSES.

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 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 fluorescein 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.

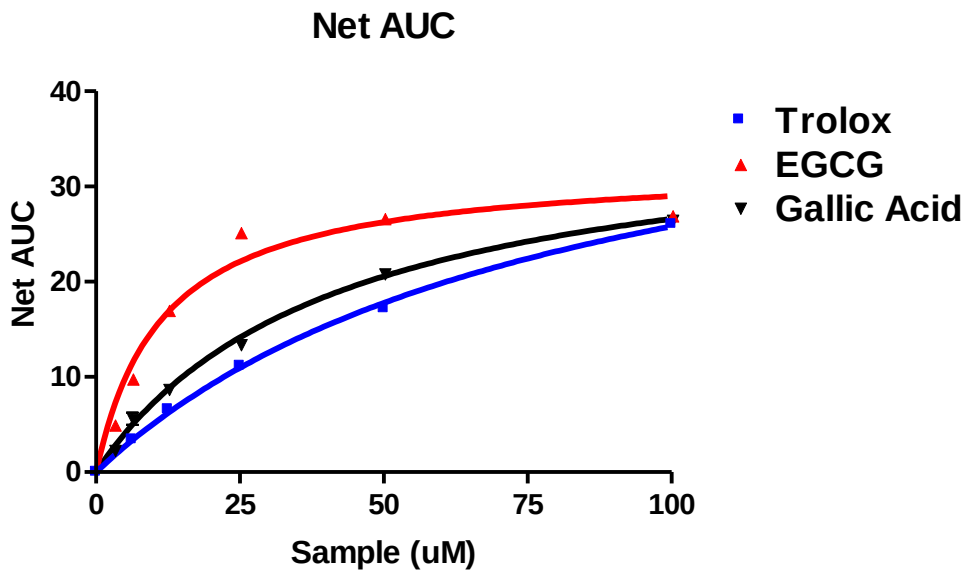
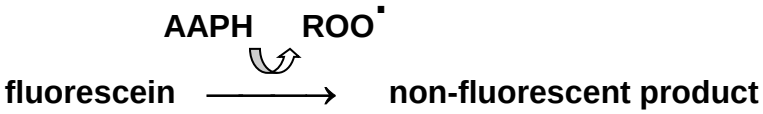


Figure 1. Effects of antioxidants in ORAC assay

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	UNIT	QTY	STORAGE
Blank Assay Plates	96-well assay plates, black	PLATE	5	RT*
AOX Assay Buffer	250 ml	BOTTLE	1	RT*
AAPH	650 mg	AMBER BOTTLE	1	-20°C
Trolox (AOX-2RB)	1.5mM in Dilution Buffer	100 µl /AMBER BOTTLE	1	-20°C
Fluorescein Solution	60X stock	6.5 ml / AMBER BOTTLE	1	+4°C
Tray	Reagent reservoir tray for multi-channel pipetters, clear or white	EACH	5	RT*

*RT= room temperature

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

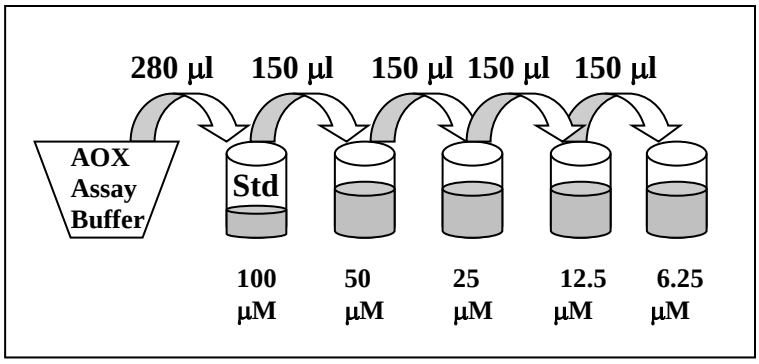
THIS BULK KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 60 WELLS PER 96 WELL PLATE FOR A TOTAL OF 300 WELLS AVAILABLE. AT LEAST 6 OF THESE WELLS ARE REQUIRED FOR TROLOX STANDARDS. THIS KIT IS DESIGNED TO BE USED IN A SINGLE USE.

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 **11.8 ml** of AOX Assay Buffer to an empty tube (not provided) and adding **0.2 ml** 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 **1.4 ml** of AOX Assay Buffer into the 1.5 mM Trolox standard amber bottle 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 10.0 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

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

Kinetic RLU Values

	Concentration uM							
	50	25	12.5	6.25	3.125	1.5625	0.78125	0
0	966.56	974.716	971.314	959.558	950.504	965.979	936.441	781.506
1	971.601	989.643	967.724	962.317	947.52	965.055	934.329	768.477
2	976.741	978.416	973.467	967.572	959.765	966.599	934.266	732.977
3	969.81	974.563	968.87	951.566	946.04	960.243	927.229	714.321
4	962.41	980.073	976.047	967.674	941.624	935.023	892.304	669.593
5	964.54	978.611	959.138	959.404	941.988	912.143	858.614	623.188
6	970.24	977.499	972.722	959.086	937.274	893.236	827.132	566.26
7	968.594	980.047	962.207	949.074	911.596	845.072	793.373	513.437
8	970.82	984.309	954.42	948.619	872.599	792.937	752.615	463.655
9	967.92	979.537	943.333	935.229	833.362	741.794	692.278	408.376
10	973.728	977.452	921.212	892.744	786.836	677.572	634.163	348.18
11	970.462	971.207	898.306	850.655	719.552	625.463	567.105	302.142
12	972.889	978.234	855.698	789.935	665.862	560.411	510.335	252.677
13	974.888	983.876	824.175	735.212	602.743	495.683	452.619	205.499
14	959.879	979.852	789.163	666.95	542.735	436.068	389.047	168.797
15	959.84	965.068	744.713	598.298	477.779	371.616	342.87	132.739
16	970.71	959.634	714.138	527.254	419.26	315.949	281.279	102.604
17	970.624	933.286	669.927	472.357	362.445	270.327	232.519	77.376
18	960.514	894.32	631.93	401.488	309.286	223.2	192.426	59.31
19	965.153	850.58	591.332	345.356	261.168	179.92	151.049	42.696
20	962.63	795.528	554.628	287.3	215.321	146.718	119.395	31.323
21	972.371	723.589	525.516	232.894	174.069	115.013	88.37	23.757
22	959.124	642.273	490.177	187.087	138.661	86.588	66.559	17.888
23	949.111	554.177	453.872	144.854	104.357	67.21	49.726	12.229
24	940.463	477.995	433.599	109.78	81.168	48.246	33.436	9.18
25	899.635	402.11	397.627	79.592	57.419	34.411	23.618	8.609
26	802.935	336.081	365.829	57.375	42.75	25.458	17.828	7.215
27	703.126	267.885	330.226	42.413	32.842	17.978	12.951	5.882
28	587.867	218.682	306.49	30.118	23.509	12.733	10.122	5.59
29	490.685	167.628	276.067	20.881	14.451	10.882	8.546	5.89
30	390.92	128.386	243.915	15.784	12.421	8.687	6.959	5.686

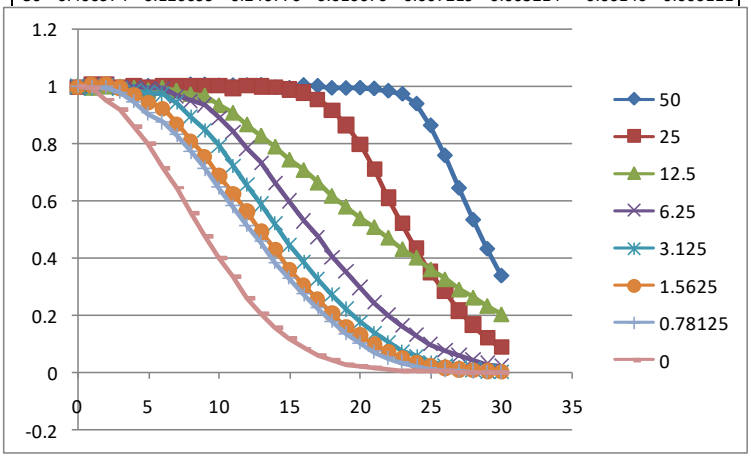
Normalized to Time=0 by (RLU/RLU0)

	Concentration uM							
	50	25	12.5	6.25	3.125	1.5625	0.78125	0
0	1	1	1	1	1	1	1	1
1	1.005246	1.015403	0.996283	1.002892	0.996842	0.999038	0.997731	0.983208
2	1.010595	1.003818	1.002229	1.008401	1.009801	1.000646	0.997663	0.937455
3	1.003382	0.999842	0.997469	0.991622	0.995276	0.994027	0.990104	0.913411
4	0.995681	1.005528	1.004901	1.008508	0.990602	0.967767	0.952584	0.855765
5	0.997898	1.004019	0.987392	0.999839	0.990987	0.943943	0.916391	0.795957
6	1.00383	1.002872	1.001458	0.999505	0.985999	0.924256	0.88257	0.722588
7	1.002117	1.005501	0.99057	0.98901	0.958823	0.874105	0.846302	0.654508
8	1.004433	1.009899	0.982506	0.988533	0.917552	0.819819	0.802516	0.590349
9	1.001415	1.004975	0.971026	0.974497	0.876028	0.766566	0.737696	0.519104
10	1.007459	1.002823	0.948119	0.929961	0.826789	0.699695	0.675264	0.441523
11	1.004061	0.996379	0.9244	0.885841	0.755582	0.645436	0.603223	0.382188
12	1.006586	1.00363	0.880279	0.82219	0.698761	0.5777	0.542235	0.318437
13	1.008666	1.009452	0.847637	0.764826	0.631962	0.510302	0.480231	0.257633
14	0.993048	1.0053	0.811382	0.69327	0.568455	0.448227	0.411936	0.210331
15	0.993007	0.990045	0.765354	0.621304	0.499711	0.381116	0.362328	0.163859
16	1.004319	0.984437	0.733693	0.546831	0.43778	0.323153	0.296161	0.12502
17	1.004229	0.95725	0.687913	0.489285	0.377652	0.275648	0.243778	0.092506
18	0.993708	0.917042	0.648567	0.414995	0.321393	0.226577	0.200707	0.069222
19	0.998536	0.871908	0.606527	0.356154	0.27047	0.181512	0.156255	0.04781
20	0.99591	0.815102	0.56852	0.295296	0.22195	0.14694	0.12225	0.033152
21	1.006047	0.74087	0.538375	0.238264	0.178292	0.113927	0.08892	0.023401
22	0.992262	0.656963	0.501781	0.190246	0.14082	0.084329	0.065488	0.015837
23	0.981842	0.566059	0.464187	0.145975	0.104515	0.064152	0.047404	0.008544
24	0.972843	0.487449	0.443194	0.109208	0.079974	0.044405	0.029904	0.004614
25	0.930356	0.409146	0.405945	0.077563	0.05484	0.03	0.019357	0.003878
26	0.829728	0.341013	0.373018	0.054274	0.039316	0.020677	0.013137	0.002081
27	0.725864	0.270644	0.336151	0.03859	0.02883	0.012889	0.007897	0.000363
28	0.605922	0.219873	0.311573	0.025701	0.018953	0.007427	0.004858	-1.3E-05
29	0.504792	0.167192	0.280069	0.016019	0.009367	0.0055	0.003165	0.000374
30	0.400974	0.126699	0.246776	0.010676	0.007219	0.003214	0.00146	0.000111

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:

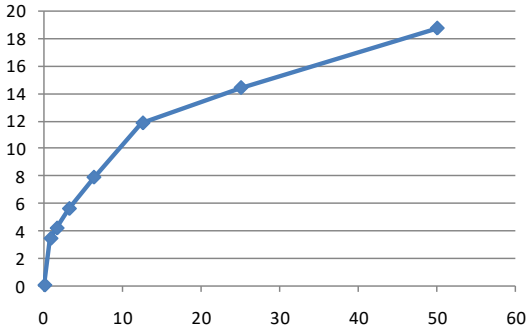
AUC= 0.5 + (F1/F0) + (F2/F0) + …+ 0.5*(F30/F0)
Where F0= normalized fluorescence at t=0

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



	50	25	12.5	6.25	3.125	1.5625	0.78125	0
AUC	27.84	23.51	20.94	16.99	14.71	13.29	12.54	9.139
Net AUC	18.701	14.371	11.801	7.851	5.571	4.151	3.401	0

Data for unknowns may be expressed as μM Trolox equivalents.



APPENDIX A: 96 well 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

ORAC ASSAY

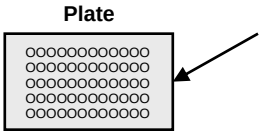
Make necessary test compound dilutions in Assay Buffer.

↓

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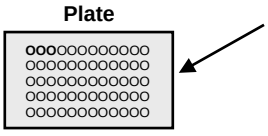
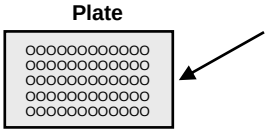
Add 25 µl/well trolox standards and test samples to fluorescein containing wells and place in incubator at 37°C for 10 minutes.



↓

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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FREQUENTLY ASKED QUESTIONS

1. **My fluorescence values are lower than those in the sample data but still generate a good Trolox standard curve. Is this cause for concern?** No. 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.

3. **I will not need all 300 wells. Can I save the reagents for later use?** No. This kit is designed for a single experiment for up to 300 wells. If you need fewer wells for your experiment, please consider ordering our single 60 well kit catalog # AOX-2 as an alternative.

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