HORAC Antioxidant Assay Kit
(60 point kit)
Cat# AOX-6

INSTRUCTION MANUAL ZBM0118.00

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 and Radical Initiator solution
Remove from box and store at 4°C
Gallic Acid Standard and Fenton Reagent
Remove from box and store at -20°C
AOX-6 Assay Buffer and black assay plate
Store at Room Temperature
Long-term Storage
Reagents are good for at least 3 months upon 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

- **Zen-Bio, Inc.**
- **3920 South Alston Ave**
- **PO Box 13888**
- **Durham, NC 27713**
- **Telephone** (919) 547-0692
- **Facsimile (FAX)** (919) 547-0693
- **Toll Free** **1-866-ADIPOSE (866)-234-7673**
- **Electronic mail (e-mail)** **information@zen-bio.com**
- **World Wide Web** **http://www.zenbio.com**

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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 HORAC (Hydroxyl (HO•) 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 hydroxyl-radical formation by the mixture of hydrogen peroxide and oxidizable metal ions (Co(II)). Gallic Acid [3,4,5-Trihydroxybenzoic acid], a simple phenolic compound, serves as a positive control inhibiting fluorescein decay in a dose dependent manner. The HORAC 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 Gallic Acid units to quantify the composite antioxidant activity present. This assay measures antioxidant activity by hydrogen atom transfer and when combined with Zen-Bio's other antioxidant assay kits, provides a comprehensive analysis of a test sample's antioxidant activity.

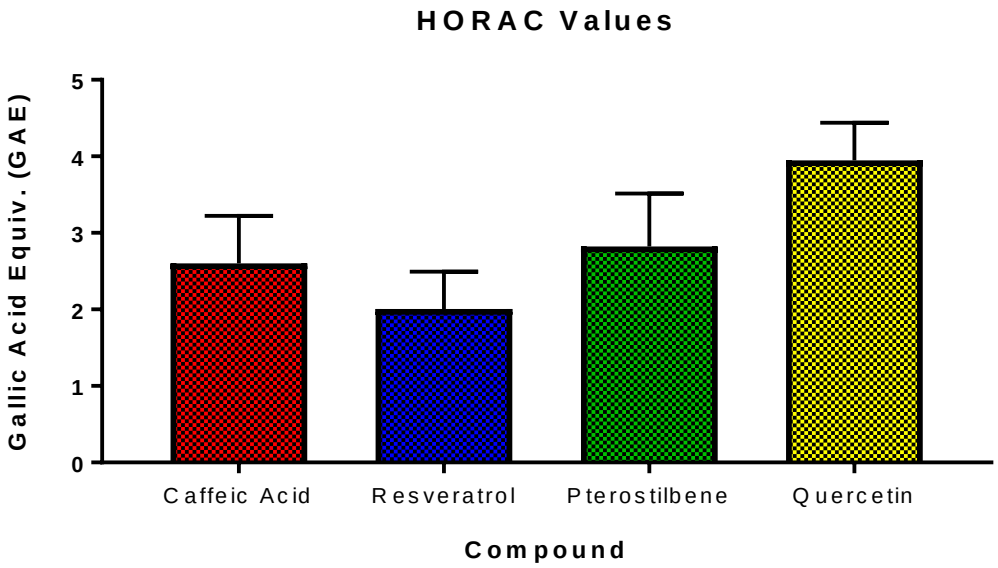
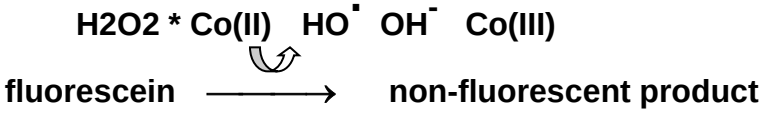


Figure 1. Effects of antioxidants in HORAC assay

Caffeic Acid, Resveratrol, Pterostilbene and Quercetin were tested for their antioxidant activity in the HORAC antioxidant assay.

PRINCIPLE OF THE ASSAY

Hydroxyl radicals (HO•) are formed from the mixture of oxidizable cobalt(II) ions and hydrogen peroxide in solution. The hydroxyl 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 45 minutes by excitation at 485 nm, emission at 535 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, gallic acid.



[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	35 ml	---	BOTTLE	1	RT
Radical Initiator	53.3x stock solution	---	100 µl /VIAL	1	4°C
Gallic Acid Solution	5mM in AOX-6 Buffer	---	200 µl /VIAL	1	-20°C
Fenton Reagent	2 ml metal ion solution	---	BOTTLE		-20°C
Fluorescein Solution	47.3x stock		300 µl /VIAL	1	4°C
Tray	For multi-channel pipettes, 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 Gallic Acid equivalents (GAE) per cell number (i.e. µmole GA/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 Gallic Acid equivalents (GAE) per gram of starting sample (i.e. µmole GA/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 Gallic Acid equivalents (GAE) per volume sample (i.e. µmole GA/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 Gallic Acid equivalents (GAE) per volume sample (i.e. µmole GA/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 micromole Gallic Acid equivalents (GAE) per volume sample (i.e. µmole GA/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 Gallic Acid equivalents (GAE) per gram of starting sample (i.e. µmole GA/g)

ASSAY PROCEDURE

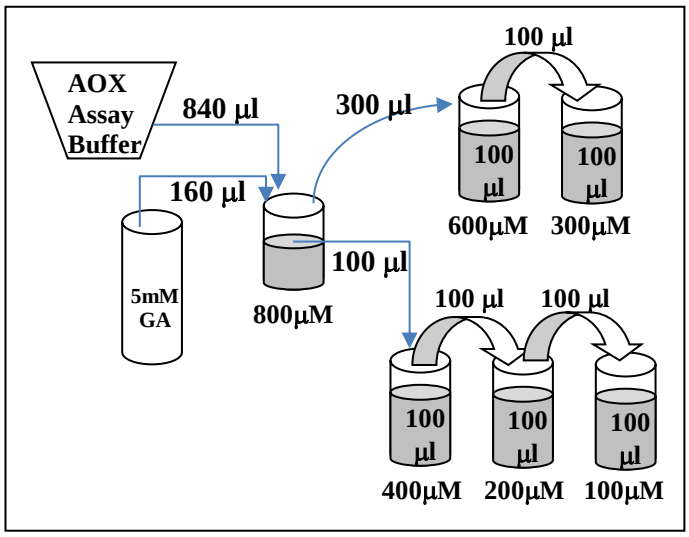
THIS KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 60 WELLS. AT LEAST 6 OF THESE WELLS ARE REQUIRED FOR GALLIC ACID STANDARDS

1. Equilibrate the plate reader incubation chamber to 37°C before beginning. Set-up plate reader to perform a kinetic read for 45 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 **11.75ml** of AOX Assay Buffer to an empty tube (not provided) and adding **0.254ml** stock fluorescein solution. Mix and protect from light.

3. Prepare Gallic acid standards as follows:

   Briefly spin down the contents of the 5 mM Gallic acid standard tube after thawing. Pipette **160 µl** of the 5 mM Gallic acid standard solution into a tube containing **840 µl** AOX Assay Buffer and mix well by vortexing. This produces a diluted stock Gallic Acid standard of **800 µM**. Pipette **100 µl** of AOX Assay Buffer into 5 tubes (not provided). Using the table and diagram below, prepare Gallic Acid standards 600, 400, 300, 200 and 100 µM. Mix each new dilution thoroughly before proceeding to the next. The **800 µM** standard dilution serves as the highest standard, and assay buffer serves as the zero standard (or blank).



Final Conc. (µM)	Buffer (µL)	Volume (µL)	GA Solution
800	840	160	5mM Stock
600	100	300	800 µM Std
400	100	100	800 µM Std
300	100	100	600 µM Std
200	100	100	400 µM Std
100	100	100	200 µM Std

4. Add 140 µl of the working fluorescein solution to each of the **INNER 60 WELLS** of the assay plate provided. Fill the outer wells with 200 µl water to maintain a constant temperature for all wells.

5. Add 20 µl of samples or Gallic acid standards (with zero standard) to individual wells of the assay plate provided. 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 Radical Initiator Working Solution by adding 37.5 µl of the concentrated Radical Initiator solution to 1.9625 mL of water.

7. After the assay plate has equilibrated to 37°C, add 20 µl of the Radical Initiator Working Solution to each inner 60 assay well.
8. To begin the assay, add 20 µl of the Fenton Reagent to each of the wells containing standards and samples from step 5. Place the assay plate in the 37°C plate reader and begin kinetic fluorescence reading.

GALLIC ACID STANDARD CURVE

Generate standard curve: see example below (Collected using a SpectraMax iD3)

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

Kinetic RLU Values

	Gallic Acid						
Time (min)	800	600	400	300	200	100	0
0	3204677	3224222	3275543	3165677	3174140	3196064	2706418
1	3198845	3221342	3194853	3174483	3201116	3143567	2509882
2	3158966	3193611	3178068	3130211	3120474	3118326	2351955
3	3114410	3139431	3139222	3116647	3105044	3043751	2144727
4	3114958	3120308	3111903	3094643	3076547	3029441	1993790
5	3079319	3107630	3070934	3092071	3034362	2907369	1865437
6	3092500	3095182	3069419	3019674	3053490	2844406	1684772
7	3086678	3086251	3043584	3039228	2969976	2750370	1541569
8	3060684	3040795	3024565	3005937	2870750	2630228	1400518
9	3019969	3008712	2988459	2929144	2800988	2547167	1312548
10	2970267	3019721	2921819	2924410	2746422	2457060	1190740
11	3018399	3024731	2847319	2803394	2636318	2333812	1080637
12	2964877	2979408	2785634	2732468	2503402	2240730	1001914
13	2965813	2971425	2720400	2649618	2405662	2117228	902737
14	2944946	2935978	2608636	2536935	2313061	2037318	820416
15	2918394	2874385	2543448	2403096	2187728	1943101	757484
16	2884539	2826559	2510472	2367278	2118385	1818681	684150
17	2857641	2742356	2406208	2241843	1966486	1716607	620368
18	2837963	2657552	2301753	2143597	1892621	1593519	557591
19	2830490	2617374	2237390	2023861	1736858	1511692	509242
20	2746710	2507688	2129011	1954257	1649878	1409292	460306
21	2687219	2433790	2066244	1811573	1515258	1290576	416120
22	2601844	2379419	1995165	1774013	1416449	1175981	376705
23	2591411	2301738	1923422	1656120	1289339	1100343	340929
24	2493928	2197819	1856615	1528304	1192552	1002876	315324
25	2420286	2143348	1767766	1450692	1090751	914225	293514
26	2351296	2050256	1666331	1323560	983730	848707	257609
27	2256073	1978252	1610924	1218451	909005	781303	249885
28	2208555	1913771	1549019	1152475	839003	713857	205778
29	2130508	1866158	1485180	1067767	745696	642048	198210
30	2036790	1783387	1409028	979406	685441	579929	185988
31	1984731	1725004	1336560	901929	634238	545480	168408
32	1898873	1638496	1248135	826618	586490	498175	149097
33	1815739	1602074	1185218	777166	539318	444942	137550
34	1758944	1535198	1110415	694075	491303	410974	128998
35	1698724	1456856	1073889	640275	458252	393768	118984
36	1597989	1400837	985664	592100	423791	350258	116617
37	1577522	1331798	937854	541615	383369	313551	107429
38	1497900	1279581	882389	501291	360312	284390	95877
39	1429658	1217176	825412	467753	325538	264675	92324
40	1365810	1165163	799255	431389	299162	246017	85533
41	1293853	1106094	714790	396637	284865	223367	75600
42	1194964	1059070	672640	366641	255695	195378	72369
43	1143408	1013720	640136	326683	239638	192745	70375
44	1068377	957570	595963	290874	224546	160851	63976
45	1016814	909813	535757	263591	206540	146371	56809

Normalized to Time=0 by (RLU/RLU0)

	Gallic Acid						
Time (min)	800	600	400	300	200	100	0
0	1	1	1	1	1	1	1
1	0.99818	0.999107	0.975366	1.002782	1.008499	0.983574	0.927382
2	0.985736	0.990506	0.970242	0.988797	0.983093	0.975677	0.869029
3	0.971833	0.973702	0.958382	0.984512	0.978232	0.952344	0.79246
4	0.972004	0.967771	0.950042	0.977561	0.969254	0.947866	0.73669
5	0.960883	0.963839	0.937534	0.976749	0.955964	0.909672	0.689264
6	0.964996	0.959978	0.937072	0.953879	0.96199	0.889972	0.62251
7	0.963179	0.957208	0.929185	0.960056	0.935679	0.860549	0.569598
8	0.955068	0.94311	0.923378	0.94954	0.904418	0.822958	0.51748
9	0.942363	0.933159	0.912355	0.925282	0.88244	0.79697	0.484976
10	0.926854	0.936574	0.892011	0.923787	0.865249	0.768777	0.439969
11	0.941873	0.938127	0.869266	0.885559	0.830561	0.730214	0.399287
12	0.925172	0.92407	0.850434	0.863154	0.788687	0.70109	0.370199
13	0.925464	0.921594	0.830519	0.836983	0.757894	0.662449	0.333554
14	0.918953	0.9106	0.796398	0.801388	0.728721	0.637446	0.303137
15	0.910667	0.891497	0.776497	0.75911	0.689235	0.607967	0.279884
16	0.900103	0.876664	0.766429	0.747795	0.667389	0.569038	0.252788
17	0.89171	0.850548	0.734598	0.708172	0.619533	0.5371	0.229221
18	0.885569	0.824246	0.702709	0.677137	0.596263	0.498588	0.206025
19	0.883237	0.811785	0.683059	0.639314	0.54719	0.472986	0.188161
20	0.857094	0.777765	0.649972	0.617327	0.519787	0.440946	0.170079
21	0.83853	0.754846	0.63081	0.572255	0.477376	0.403802	0.153753
22	0.81189	0.737982	0.60911	0.56039	0.446247	0.367947	0.13919
23	0.808634	0.713889	0.587207	0.523149	0.406201	0.344281	0.125971
24	0.778215	0.681659	0.566811	0.482773	0.375709	0.313785	0.11651
25	0.755236	0.664764	0.539686	0.458256	0.343637	0.286047	0.108451
26	0.733708	0.635892	0.508719	0.418097	0.30992	0.265548	0.095184
27	0.703994	0.613559	0.491804	0.384894	0.286378	0.244458	0.092331
28	0.689166	0.593561	0.472904	0.364053	0.264325	0.223355	0.076033
29	0.664812	0.578793	0.453415	0.337295	0.234929	0.200887	0.073237
30	0.635568	0.553122	0.430166	0.309383	0.215945	0.181451	0.068721
31	0.619323	0.535014	0.408042	0.284909	0.199814	0.170672	0.062225
32	0.592532	0.508183	0.381047	0.261119	0.184771	0.155871	0.05509
33	0.56659	0.496887	0.361839	0.245498	0.16991	0.139216	0.050824
34	0.548868	0.476145	0.339002	0.21925	0.154783	0.128588	0.047664
35	0.530077	0.451847	0.327851	0.202255	0.14437	0.123204	0.043964
36	0.498643	0.434473	0.300916	0.187037	0.133514	0.10959	0.043089
37	0.492256	0.41306	0.28632	0.17109	0.120779	0.098105	0.039694
38	0.467411	0.396865	0.269387	0.158352	0.113515	0.088981	0.035426
39	0.446116	0.37751	0.251992	0.147758	0.102559	0.082813	0.034113
40	0.426193	0.361378	0.244007	0.136271	0.09425	0.076975	0.031604
41	0.403739	0.343058	0.21822	0.125293	0.089746	0.069888	0.027934
42	0.372881	0.328473	0.205352	0.115818	0.080556	0.061131	0.02674
43	0.356794	0.314408	0.195429	0.103195	0.075497	0.060307	0.026003
44	0.333381	0.296993	0.181943	0.091884	0.070742	0.050328	0.023639
45	0.317291	0.282181	0.163563	0.083265	0.06507	0.045797	0.02099

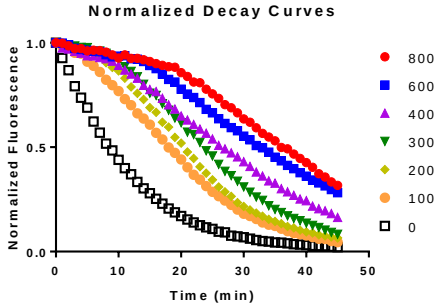
Use normalized data to generate Area Under the Curve (AUC) values. AUC values can be calculated by a statistical program (such as GraphPad Prism) or by the following formula:

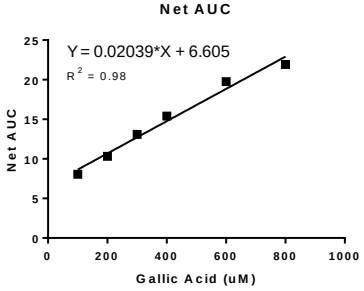
AUC= 0.5 + (F1/F0) + (F2/F0) + …+ 0.5*(F30/F0)
Where F0= normalized fluorescence at t=0

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

	800	600	400	300	200	100	0
AUC	33.4	31.3	26.9	24.6	21.8	19.5	11.5
Net AUC	21.9	19.8	15.4	13.1	10.3	8.1	0

Data for unknowns may be expressed as
µM Gallic Acid Equivalents or µmole GA/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

HORAC ASSAY

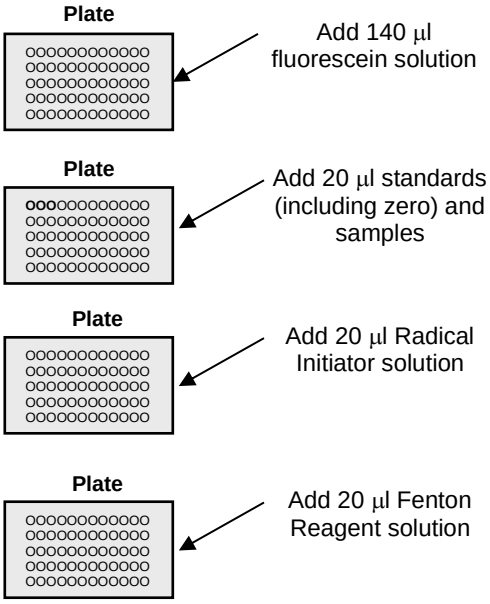
Make necessary test compound dilutions in Assay Buffer.

↓

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

↓

Add 140 µl/well Working Fluorescein Solution to blank assay plate.



↓

Add 20 µl/well gallic acid standards and test samples to fluorescein containing wells and place in incubator at 37°C for 10 minutes.

↓

Prepare Radical Initiator solution. Add 20 µl/well of Radical Initiator working solution.

↓

Add 20 µl/well of Fenton Reagent Solution and place assay plate in plate reader. Begin kinetic fluorescence read.
Excitation= 485 nm; Emission=528 - 538 nm;
(Cutoff=530 nm, if necessary)

REFERENCES

1. J Agr and Food Chem. 50: 2772-2777, 2002.
2. Food Control, 21:518-523, 2010.

FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my fluorescence values are lower than those in the sample data but still generate a good Gallic Acid 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 SpectrMax iD3 fluorimeter, other instruments vary in sensitivity and can give lower values. If the Gallic Acid 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 Gallic Acid 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.