NORAC Antioxidant Assay Kit
(60 point kit)
Cat# AOX-7

INSTRUCTION MANUAL ZBM0121.00

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier on dry ice.
All orders must be processed immediately upon arrival. Any adverse conditions upon arrival must be reported within 7 days.

DHR-123 and Radical Initiator Solutions
Remove from box and store at -80°C
Trolox Standard
Remove from box and store at -20°C
AOX Assay Buffer and black assay plate
Store at Room Temperature
Long-term Storage
If properly stored, 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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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.

Peroxynitrite is a cytotoxic species that has been associated with Alzheimer's disease, rheumatoid arthritis, cancer and atherosclerosis, and can be generated by nitric oxide and superoxide in endothelial cells, Kupffer cells, macrophages and neutrophils. The Zen-Bio NORAC (Peroxynitrite [ONOO$^-$] Radical Absorbance Capacity) Antioxidant Assay Kit can be used to determine the total peroxynitrite scavenging capacity of biological fluids, cells, and tissue, as well as naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The *in vitro* assay measures the increase in rhodamine-123 fluorescence over time due to the oxidation of dihydrorhodamine-123 by peroxynitrite radicals formed through SIN-1 (3-morpholinosydnonimine) degradation. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control inhibiting DHR123 oxidation in a dose dependent manner. The NORAC assay can be performed as an endpoint or kinetic assay measuring antioxidant inhibition of rhodamine-123 fluorescence over time. The antioxidant activity in samples is normalized to equivalent Trolox units to quantify the composite antioxidant activity present.

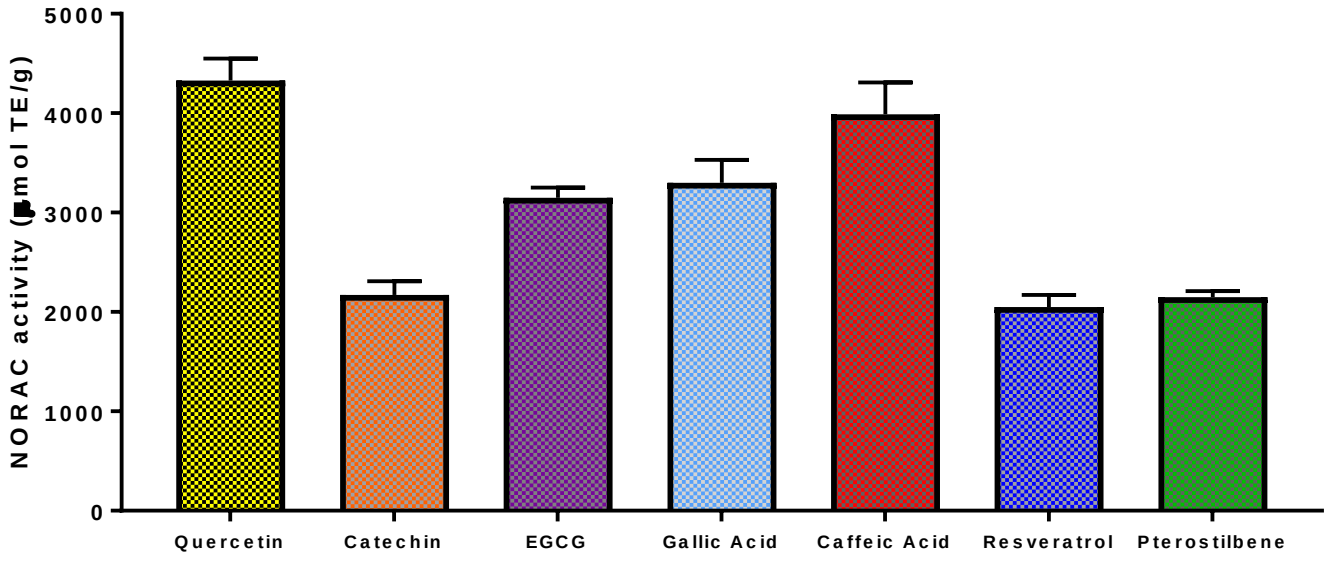
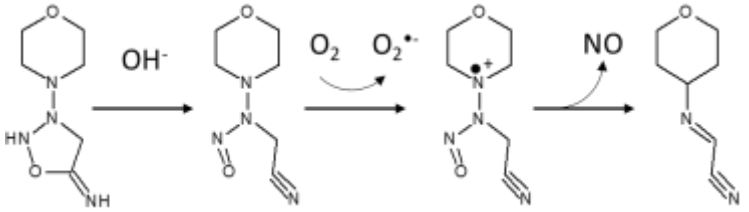


Figure 1. Effects of antioxidants in the NORAC assay

Qurecetin, Catechin, Epigallocatechin gallate (EGCG), Gallic acid, Caffeic acid, Resveratrol and Pterostilbene were tested for their antioxidant activity in the NORAC antioxidant assay.

PRINCIPLE OF THE ASSAY

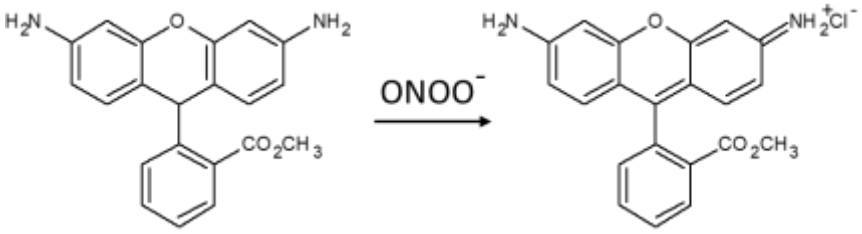
Peroxynitrite ($ONOO^-$) is formed from the superoxide and nitric oxide created by breakdown of SIN-1 (3-morpholinosydnonimine) at 37 °C.



SIN-1

$$O_2^{\bullet-} + {}^{\bullet}NO \longrightarrow ONOO^-$$

Peroxynitrite can oxidize Dihydrorhodamine-123 (DHR-123) to generate Rhodamine-123, a highly fluorescent compound. Antioxidants suppress this reaction, inhibiting the oxidation of DHR-123 and the increase in fluorescence signal. The fluorescence signal is measured over 30 minutes by excitation at 485 nm, emission at 535 nm. The concentration of antioxidant in the test sample is inversely proportional to the fluorescence intensity and is assessed by comparing the area under the curve to that of a known antioxidant, trolox.



Dihydrorhodamine 123
No fluorescence

Rhodamine 123
Fluorescence

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	UNIT	QTY	STORAGE
Blank Assay Plates	96-well assay plates, black clear bottom	PLATE	1	-----
AOX-7 Assay Buffer	30 ml	BOTTLE	1	RT
Radical Initiator	10 µl	VIAL	1	-80°C
Trolox Solution	1.5 mM solution	20 µl /VIAL	1	-20°C
DHR-123 Solution	1000x stock	20 µl /VIAL	1	-80°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 or clear 96-well plate for preparing standards and working solutions
- Fluorescence plate reader able to perform excitation=485nm; emission=528 -538nm
- Fluorescence plate reader with incubator chamber set to 37°C.
- Nitrogen gas to purge buffer prior to assay.

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-7 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

Prior to using the Assay Buffer it is best to purge it with nitrogen gas for at least 5 minutes.

Place DHR-123 and Radical Initiator solutions on ice prior to making working solutions.

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 2 minute intervals. Excitation = 485 nm; Emission = 528 - 538 nm. **SET PLATE READER TO BOTTOM READ.**
2. 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-7 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**.

   Prepare the following standards in tubes or a clear 96-well plate (not provided) using Assay Buffer as diluent. Mix each new dilution thoroughly before proceeding to the next.

Standard	Final Conc. (µM)	Buffer (µL)	Volume (µL)	Trolox Solution
Std 30	30	70	30	100 µM Stock
Std 20	20	160	40	100 µM Stock
Std 10	10	100	100	Std 20
Std 5	5	100	100	Std 10
Std 2	2	60	40	Std 5
Std 0	0	100	0	-

3. Using the **inner 60 wells**, add 25 µl of prepared 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. Trolox standards should be tested in duplicate and samples in triplicate. [**IF THE AOX ACTIVITY OF THE TEST SAMPLES IS UNKNOWN, WE RECOMMEND PREPARING SEVERAL DILUTIONS IN AOX ASSAY BUFFER.]**
4. Prepare DHR-123 working solution from the stock solution provided by transferring **10.0ml** of AOX Assay Buffer to an empty tube (not provided) and adding **10 µl** stock DHR-123 solution. Mix and protect from light.
5. Add 150 µl of the working DHR-123 solution to each of the **inner 60 wells** containing standards, samples and negative control.
6. Add 25 µl of assay buffer to the negative control wells (NO RADICAL INITIATOR).
7. Prepare the Radical Initiator Working Solution by adding **2.0 ml** AOX Assay Buffer to a tube (not provided) and add **2 µl** stock Radical Initiator solution.
8. To begin the assay, add 25 µl of the Radical Initiator Working Solution to the remaining wells containing trolox standards and samples. Place the assay plate in the plate reader and begin kinetic fluorescence reading. Alternatively, incubate for 30 minutes at 37°C and read plate.

TROLOX STANDARD CURVE

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

The negative control RFU values have been subtracted from the trolox RFU values.

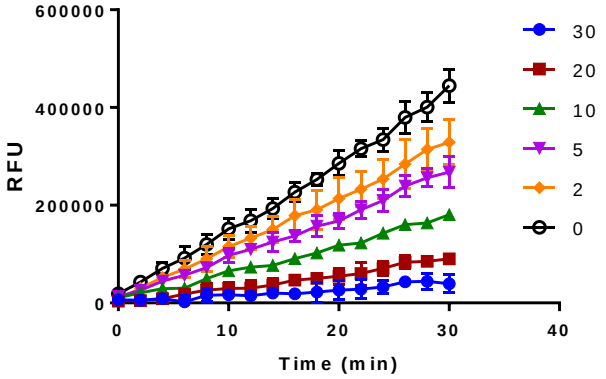
[DO NOT use this standard curve to generate your data. This is an example.]

Kinetic RFU Values

Time (min)	30		20		10		5		2		0	
0	1084.33	11030.3	-1965.7	9917.33	4071.33	17474.3	10904.3	15251.3	4152.33	11432.3	18856.3	18286.3
2	271.833	11615.8	-304.17	9591.83	16169.8	25088.8	26142.8	25036.8	34285.8	26178.8	48984.8	36131.8
4	4468.5	12783.5	4427.5	12984.5	26489.5	31955.5	47974.5	41960.5	57866.5	46214.5	79741.5	61863.5
6	-7027.8	11893.2	13819.2	22433.2	24691.2	36308.2	56805.2	57051.2	81610.2	57303.2	108539	75325.2
8	6482.5	24802.5	15812.5	36188.5	48069.5	51021.5	74503.5	70408.5	107777	72507.5	134209	105214
10	8106.83	24907.8	20733.8	38823.8	62163.8	69436.8	106858	87357.8	130889	99874.8	167050	136864
12	10757.8	19142.8	18597.8	41599.8	72684.8	73895.8	108438	111385	149281	116282	184762	151781
14	12804.3	27452.3	26930.3	47030.3	71304.3	82589.3	138707	110967	168998	131263	207595	178468
16	12429.8	24958.8	43548.8	49475.8	82347.8	98891.8	145759	128456	202511	155317	240478	212781
18	7824.17	37093.2	43803.2	57019.2	99291.2	105107	172487	142508	218751	162275	261765	243698
20	12999.5	39745.5	43064.5	66607.5	117324	118712	178174	157619	243658	183360	303597	267818
22	14834.3	41662.3	43677.3	77307.3	121751	124232	203064	178409	258738	207208	327844	303496
24	22567.5	42786.5	60742.5	81078.5	143316	142223	225636	194320	282424	225222	350692	317733
26	36253.8	50034.8	74200.8	92657.8	162051	158084	254419	224063	319991	248877	402397	357213
28	32133.2	56131.2	77905.2	92718.2	164469	163488	269534	243262	345004	283153	422047	379743
30	26263.3	52369.3	85804.3	94934.3	181240	180716	289575	245871	361650	296224	469006	420841

Use the RFU 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:

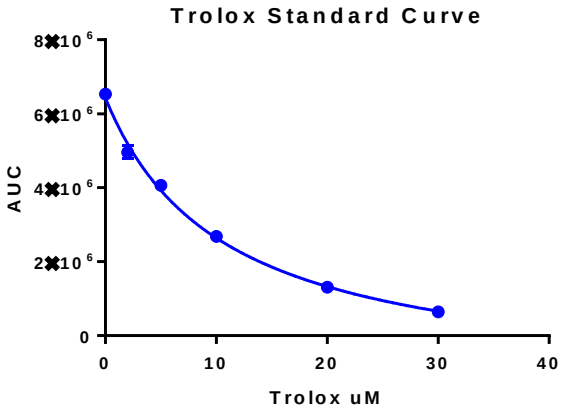
AUC= 2*(F0+F2)/2 + 2*(F2+F4)/2 + ….
Or
AUC= (F0+F2) + (F2+F4) + (F4+F6) + …
Where F0= fluorescence at t=0
Or
AUC= F0+2*F2+2*F4 +…. + F30



If the kinetic analysis is performed using different interval spans than 2 minutes, the equation for AUC should be altered to: AUC = span*(F0+F2)/2 + span*(F2+F4)/2 + ….span*(F28+F30)/2

	30	20	10	5	2	0
AUC ($x10^6$)	0.65	1.30	2.68	4.06	4.95	6.53

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								
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APPENDIX B: Protocol Flowchart

NORAC ASSAY

Make necessary test compound dilutions in nitrogen-purged Assay Buffer.

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Prior to assay, warm plate chamber to 37°C, prepare DHR-123 working solution and Trolox standards.

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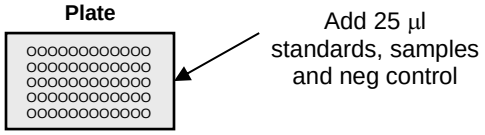
Add 25 µl/well Trolox standards, test samples and negative control to replicate wells.



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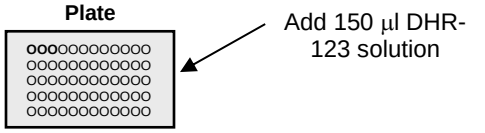
Add 150 µl/well Working DHR-123 Solution to all wells.



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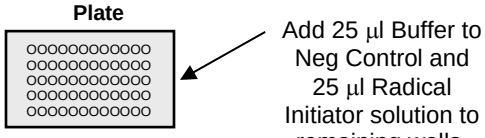
Add 25 µl Buffer to Negative Control wells.
Prepare Radical Initiator solution. Add 25 µl/well of Radical Initiator working solution and place assay plate in plate reader.
Begin kinetic fluorescence read.
Excitation= 485 nm; Emission=528 - 538 nm.
Alternatively, incubate at 37°C for 30 minutes and read fluorescence.



REFERENCES

1. Free Radical Biol Med. 16(2):149-156, 1994
2. J Agr and Food Chem. 49:3614-3621, 2001.
3. J Agr and Food Chem. 54: 8604-8610, 2006.
4. Food Science & Nutr. 2(6): 647-654, 2014.
5. Arch Biochem Biophys. 361(2): 331-339, 1999.
6. Pharmaceutical Res. 21(10):1750-1757, 2004.

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 SpectraMax iD3 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 AUC value must fall on the Trolox AUC standard curve. We recommend preparing several serial dilutions of your test sample using the AOX assay buffer to ensure that you generate usable AUC values.

3. **If I can't perform a kinetic assay, what time point should I use?** We have found that using the 30 minute time point generates robust data for the Trolox standard curve. SIN-1 degradation and DHR-123 oxidation follow close to linear kinetics which makes it possible to use the RFU values from a single time point to calculate AOX activity of your sample.