CLORAC Antioxidant Assay Kit
(96 point kit)
Cat# AOX-8

INSTRUCTION MANUAL ZBM0122.01

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
Trolox Standard
Remove from box and store at -20°C
AOX-8 Assay Buffer and black assay plate
Store at Room Temperature
Long-term Storage
If stored properly upon arrival, 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.

The Zen-Bio CLORAC (HypoChLORite (CLO^-) 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 due to oxidation by hypochlorite ions. 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 CLORAC assay is an endpoint assay measuring antioxidant protection of fluorescein oxidation after fifteen minutes. 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. All test samples must be in aqueous solutions, do not use DMSO to solubilize samples.

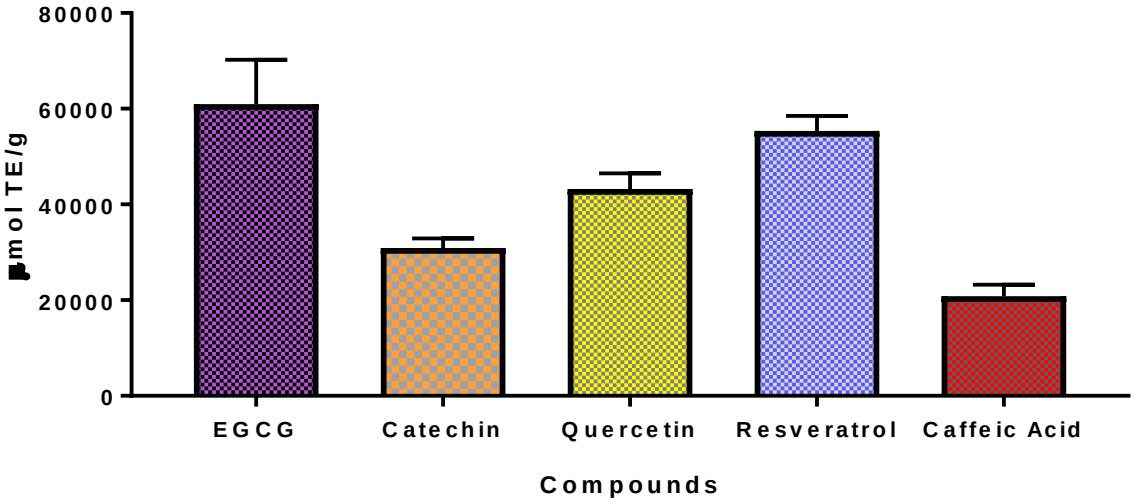
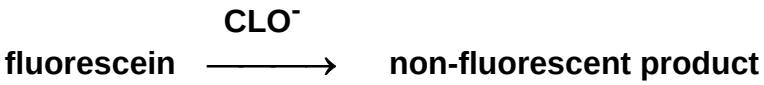


Figure 1. Effects of antioxidants in the CLORAC assay

EGCG. Catechin, Quercetin, Resveratrol and Caffeic acid were tested for their antioxidant activity in the CLORAC antioxidant assay.

PRINCIPLE OF THE ASSAY

Hypochlorite ions (CLO^-) 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 scavenging the hypochlorite ions, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured after a 15 minute incubation by excitation at 485 nm, emission at 535 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity and is assessed by comparison to that of a known antioxidant, trolox.



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	30 ml	---	BOTTLE	1	RT
Hypochlorite Solution	12,300x stock solution	---	10 µl /VIAL	1	4°C
Trolox Solution	1.5mM in AOX-8 Buffer	---	50 µl /VIAL	1	-20°C
Fluorescein Solution	20.95x stock		1 ml /VIAL	1	4°C
Tray	For multi-channel pipettes, clear polyvinyl	---	EACH	2	-----

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. µmole 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 micromole Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

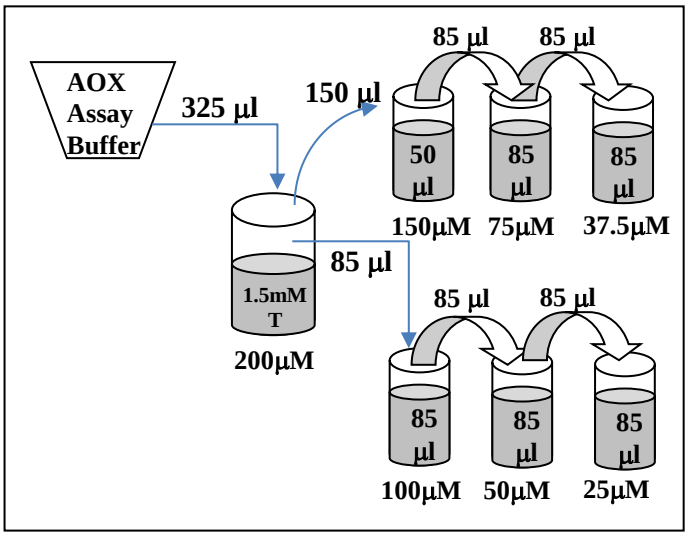
Food Extract Preparation

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. µmole TE/g)

ASSAY PROCEDURE

THIS KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 96 WELLS. AT LEAST 8 OF THESE WELLS ARE REQUIRED FOR TROLOX STANDARDS

1. Set-up plate reader to read fluorescence with 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 **15.24ml** of AOX Assay Buffer to an empty tube (not provided) and adding **0.764ml** 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 **325 µl** of AOX Assay Buffer into the 1.5 mM Trolox standard solution and mix well by vortexing. This produces a diluted stock Trolox standard of **200 µM**. Pipette **50 µl** of AOX Assay Buffer into one tube and **85 µl** into 6 tubes (not provided). Using the table and diagram below, prepare Trolox standards 150, 100, 75, 50, 37.5 and 25 µM. Mix each new dilution thoroughly before proceeding to the next. The **200 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)	Trolox Solution
200	325	50	1.5mM stock
150	50	150	200 µM
100	85	85	200 µM
75	85	85	150 µM
50	85	85	100 µM
37.5	85	85	75 µM
25	85	85	50 µM
0	85	0	-

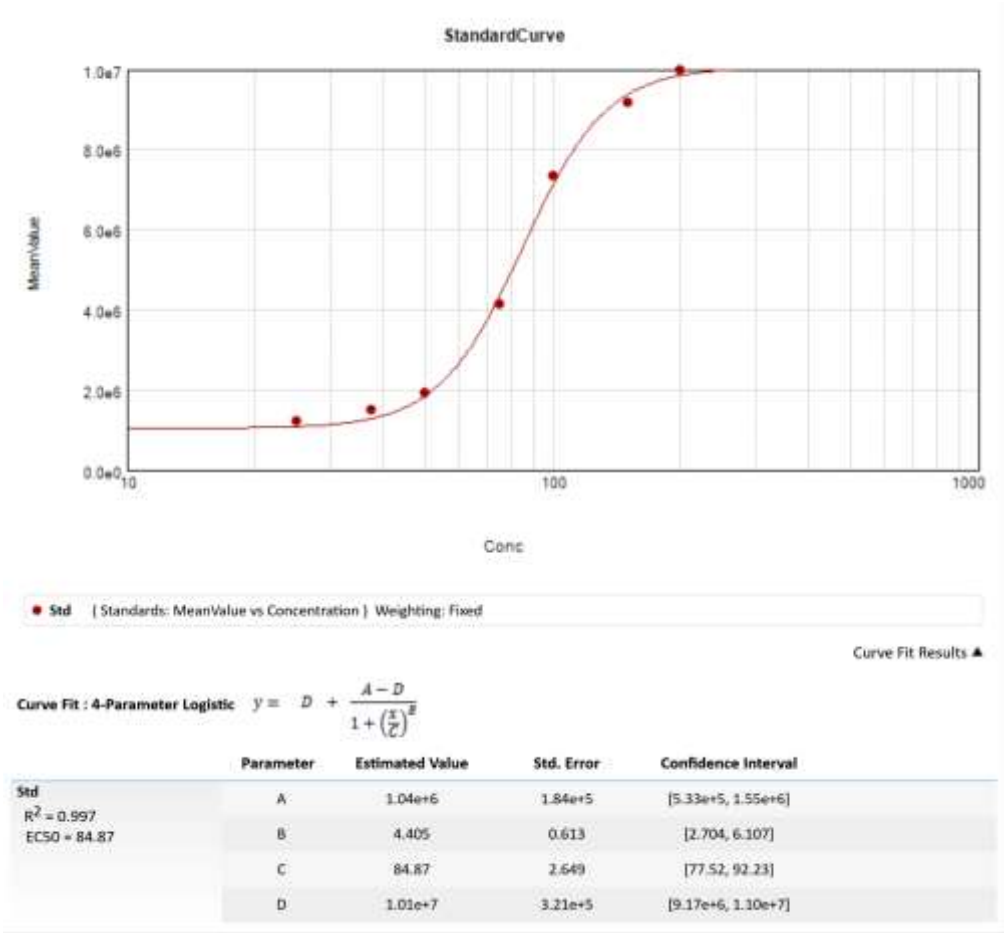
4. Add 25 µl of samples or Trolox standards (with zero standard) to the wells of the assay plate provided. [**IF THE AOX ACTIVITY OF THE TEST SAMPLES IS UNKNOWN, WE RECOMMEND PREPARING SEVERAL DILUTIONS IN AOX ASSAY BUFFER.]**
5. Prepare the Hypochlorite Working Solution using a two-step dilution process. Dilution 1) Dilute the concentrated Hypochlorite stock by adding 1 µl to 122 µL of Assay Buffer in a separate tube (not provided). In a new tube (not provided) pipette 2.97 mL of Assay Buffer and add 30 µL of Dilution 1 to create the Working Solution and mix.
6. Add 25 µl of the hypochlorite Working Solution to each well of the assay plate. Gently tap the plate to mix the samples and hypochlorite solutions.
7. Add 150 µl of the working fluorescein solution to each well of the assay plate. Incubate at room temperature for 15 minutes away from light.
8. Assess fluorescence in plate reader using excitation = 485 nm; emission = 528 - 538 nm.

TROLOX 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.]

Trolox µM	RFU 1	RFU 2	RFU 3
200	9929092	10006587	9975626
150	9411907	9244664	8842301
100	7426376	7644836	6958400
75	3536608	5361064	3519487
50	1822875	2022015	1888172
37.5	1366131	1732332	1373094
25	1190882	1285544	1163453
0	667879	775091	729846



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

APPENDIX A: Plate layout

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

APPENDIX B: Protocol Flowchart

CLORAC ASSAY

Make necessary test compound dilutions in Assay Buffer.

↓

Prior to assay, prepare fluorescein working solution and trolox standards.

↓

Add 25 µl/well trolox standards and test samples to appropriate wells.

↓

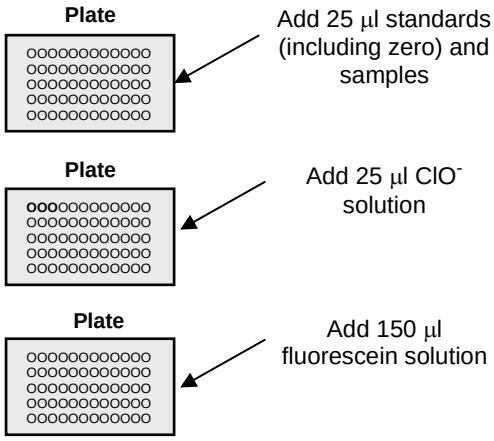
Prepare Hypochlorite solution. Add 25 µl/well of ClO^- working solution.

↓

Add 150 µl/well Working Fluorescein Solution to every well. Incubate 15 minutes at room temperature.

↓

Place plate in fluorescence plate reader and measure fluorescein fluorescence. Excitation= 485 nm; Emission=528 - 538 nm; (Cutoff=530 nm, if necessary)**.**



REFERENCES

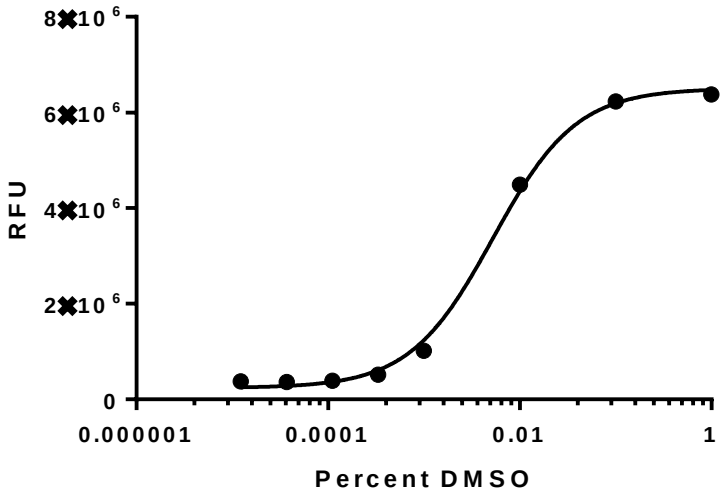
1. *Food Chemistry*. **241**: 480-492, 2018.
2. *Talanta*, **80**:2196-2198, 2010.
3. *Cell Mol Biol Letters* **15**: 90-97, 2010.

FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my fluorescence values are lower than those in the sample data but still generate a good 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 SpectrMax 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 fluorescence value must fall on the Trolox standard curve. We recommend preparing several serial dilutions of your test sample using the AOX assay buffer to ensure that you generate usable fluorescence values.

3. **What solvents interfere (react) with this assay?** DMSO readily reacts with ClO^- acting as an antioxidant in this assay and should be avoided. DMSO concentrations above 0.0001% will interfere with the assay. Ethanol, Methanol and Dimethylformamide can be used, but should be used at less than 1%.



Effect of DMSO in the CLORAC assay

Increasing concentrations of DMSO were tested for their antioxidant activity in the CLORAC antioxidant assay.