CAA Antioxidant Assay Kit
(192 point kit)
Cat# AOX-9

INSTRUCTION MANUAL ZBM0120.00

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.

Quercetin Standard, DCFH-DA Probe and Radical Initiator
Remove from box and store at -20°C
CAA Assay Buffer
Store at Room Temperature
Assay plates and Feeding Trays
Store at Room Temperature
Long-term Storage
Reagents are good for at least 6 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

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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 these systems and cause severe damage.

The Zen-Bio CAA (Cellular Antioxidant Activity) Assay Kit can be used to determine the antioxidant capacity of biological fluids, naturally occurring or synthetic compounds, dietary supplements, and therapeutics. The assay measures the capability of a test sample to inhibit intracellular ROS using an intact cell-based system. This requires that the test sample can enter the cell and maintain antioxidant activity. A cell permeant probe, dichlorodihydrofluorescin diacetate (DCFH-DA), is trapped in the cell by deacylation by cellular esterases generating DCFH which is not fluorescent. Intracellular ROS and probe oxidation are induced by peroxyl radical formation from an exogenous Radical Initiator, which produces fluorescent DCF inside the cell. Cell permeant antioxidants inhibit this oxidation reaction and reduce intracellular fluorescence. The flavonoid, quercetin, serves as a positive control and comparator to determine a sample's antioxidant activity. The CAA assay is a kinetic assay measuring increasing fluorescence and antioxidant protection over time. The antioxidant activity in the test samples can be normalized to equivalent quercetin units to quantify the composite antioxidant activity present. This assay measures cell-based antioxidant activity and when combined with Zen-Bio's other antioxidant assay kits, provides a comprehensive analysis of a test sample's antioxidant activity.

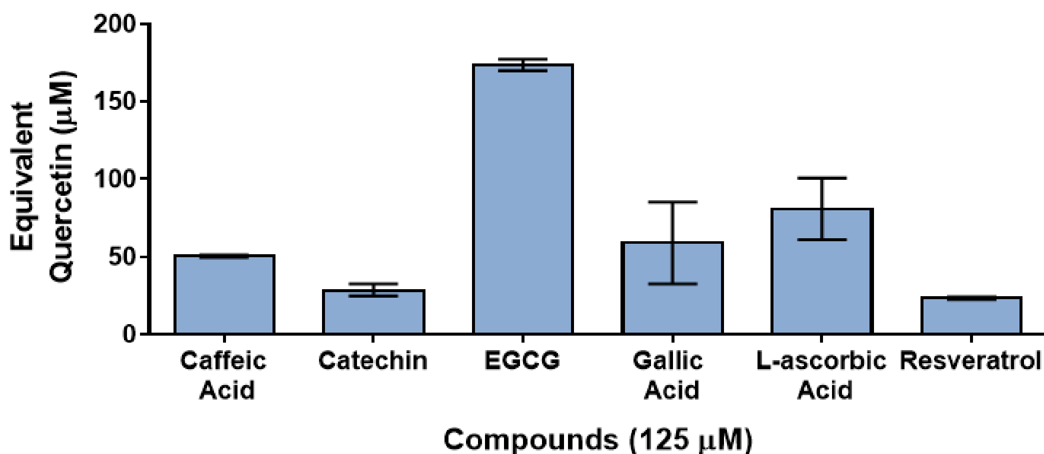
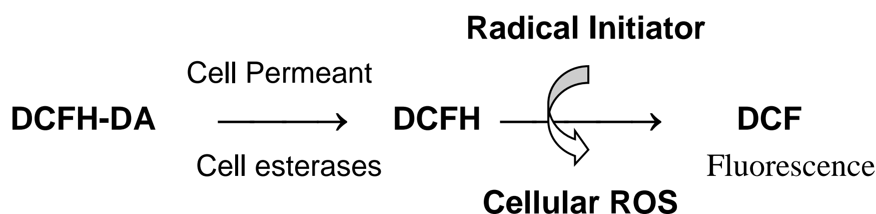


Figure 1. Effects of antioxidants in CAA assay

Caffeic Acid, Catechin, EGCG, Gallic Acid, L-ascorbic acid, and Resveretrol were tested for their antioxidant activity in the CAA assay at 125 µM.

PRINCIPLE OF THE ASSAY

The Cellular Antioxidant Activity (CAA) assay measures the capacity of antioxidants to inhibit the oxidation of the nonfluorescent probe, DCFH to the fluorescent species, DCF, by intracellular reactive oxygen species (ROS). Cellular ROS is induced by peroxyl radical formation from the Radical Initiator and leads to a gradual increase in fluorescence due to oxidation of DCFH within the cell. Antioxidants inhibit this reaction by interfering with ROS activity, leading to a reduction in cellular fluorescence over time. The fluorescence signal is measured over 60 minutes by excitation at 485 nm, emission at 538 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, quercetin.



[Antioxidants interfere with the oxidation of DCFH by cellular ROS]

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	UNIT	QTY	STORAGE
Black, Clear-bottom TC-treated Culture Plates	96-well assay plate, black, clear-bottom	PLATE	2	-----
CAA Assay Buffer	26 ml	CLEAR BOTTLE	1	Room Temp
Quercetin Standard	50 mM quercetin solution	10 µl / VIAL	1	-20°C
DCFH-DA Probe	35 µl solution	AMBER VIAL	1	-20°C
Radical Initiator	4 mg powder	ROUND BOTTLE	1	-20°C
Multichannel Pipette Reservoirs	Clear polyvinyl reservoir	EACH	2	-----

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

- Adherent cells for testing samples
- Growth medium for culturing cells
- Sterile DPBS to wash the cells between steps
- Incubator to maintain cells in culture, 37°C, 5% CO_2
- Multi-channel pipette, single channel pipette and pipette tips
- 96-well microplate or tubes for preparing standards.
- Fluorescence microplate reader with incubator plate chamber able to perform kinetic fluorescence measurement using excitation of 485nm and emission of 538nm

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 ice cold buffer or serum-free medium.
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. [Alternatively, pass supernatant through a 10 kDa molecular weight cutoff filter to reduce protein content.]
5. If not using the same day, store the samples at -80°C.
6. Data is expressed as Quercetin equivalents (QE) per cell number (i.e. µmole Q/10^6 cells)

Tissue Lysate Preparation

1. Homogenize tissue samples on ice in cold buffer or serum-free medium at ~20mg tissue / ml.
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. [Alternatively, pass supernatant through a 10 kDa molecular weight cutoff filter to reduce protein content.]
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as Quercetin equivalents (QE) per gram of starting sample (i.e. µmole Q/g)

Plasma Preparation

1. Collect the blood in a tube containing heparin as 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 Quercetin equivalents (QE) per volume sample (i.e. µmole Q/L)

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 Quercetin equivalents per volume sample (i.e. µmole Q/L)

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 Quercetin equivalents per volume sample (i.e. µmole Q/L)

Food Extract Preparation

1. Weigh the starting material and homogenize in a small volume ice cold buffer or serum-free medium. [ex. 5g of material in 100mL water].
2. Filter or centrifuge homogenates to obtain supernatant.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Quercetin equivalents per gram of starting sample (i.e. µmole Q/g)

ASSAY PROCEDURE

THIS KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 192 WELLS. AT LEAST 16 WELLS PER PLATE ARE SUGGESTED FOR QUERCETIN STANDARDS.

1. Use the included sterile black, clear-bottom TC-treated 96-well plates to culture cells (e.g. HepG2, HeLa) to 90 – 100% confluence before performing the assay. This may require seeding up to 60,000 cells per well depending on the cell type.
2. On the day of the assay, prepare the working DCFH-DA probe dilution. Briefly spin down the contents of the 12.5 mM DCFH-DA probe tube after thawing. Transfer 30 µl to 15 mL of cell growth medium, preferably without serum and phenol red (not included). Protect DCFH-DA solution from exposure to light.
3. Prepare Quercetin standards. Briefly spin down the contents of the 50 mM Quercetin standard tube after thawing. Prepare a 1 mM stock of quercetin in Assay Buffer by transferring 4 µl 50 mM Quercetin to 196 µl Serum-free Medium. Using the 1 mM quercetin stock and the table below, prepare quercetin standards 120, 100, 80, 60, 40, 20, 10 and 0 µM. Mix each dilution thoroughly. The 120 µM standard dilution serves as the highest standard, and serum-free growth medium serves as the zero standard (or blank). **[The quercetin standard concentrations are dependent on the cell type being tested and may need to be higher or lower than shown]**

The volumes shown are sufficient for duplicate samples on one plate.

Standard	1 mM Quercetin (µL)	Serum-free Medium (µL)	Total Volume (µL)	Final Concentration (µM)
1	30	220	250	120
2	25	225	250	100
3	20	230	250	80
4	15	235	250	60
5	10	240	250	40
6	5	245	250	20
7	2.5	247.5	250	10
8	0	250	250	0

4. <u>Perform the assay using 1 plate at a time</u>: Carefully remove culture medium from the wells and gently wash the cell monolayer once with 100 µl DPBS (not included).
5. Add 50 µl of the working DCFH-DA probe dilution from step 2 to each well.
6. Add 50 µl of quercetin standard dilutions from step 3 and your test samples to the appropriate wells containing the working probe dilution.
7. Protect the cells from light and incubate for 1 hour in a 37°C, 5% CO_2 incubator.

8. While the cells are incubating, equilibrate the plate reader incubation chamber to 37°C. Set-up plate reader to perform a kinetic read for 60 minutes with 5 minute intervals. Excitation = 485 nm; Emission = 538 nm. **SET PLATE READER TO BOTTOM READ.**
9. Prepare the Radical Initiator Solution by transferring 25 mL of Assay Buffer to the Radical Initiator bottle. Mix well by inversion and vortexing. This solution is stable on ice for 6 hours.
10. After the 1 hour cell incubation, carefully remove the treatments and gently wash the cell monolayer one time with 100 μl DPBS.
11. To start the assay, add 100 μl of the reconstituted Radical Initiator solution from Step 9.
12. Place the plate in the 37°C plate reader and begin kinetic fluorescence reading using ex=485nm and em=538nm. Record and save measurements every 1 to 5 minutes for a total of 60 minutes.

DATA ANALYSIS

1. Calculate the area under the curve (AUC) for each sample and standard using the relative fluorescence unit values. The AUC can be calculated using software packages or the equation below:

$$AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + \ldots + RFU_{59}/RFU_0 + RFU_{60}/RFU_0$$

RFU_0 = relative fluorescence unit at time point zero.
RFU_x = relative fluorescence unit at time points > 0
(i.e. RFU_{25} = relative fluorescence unit at 25 minutes)

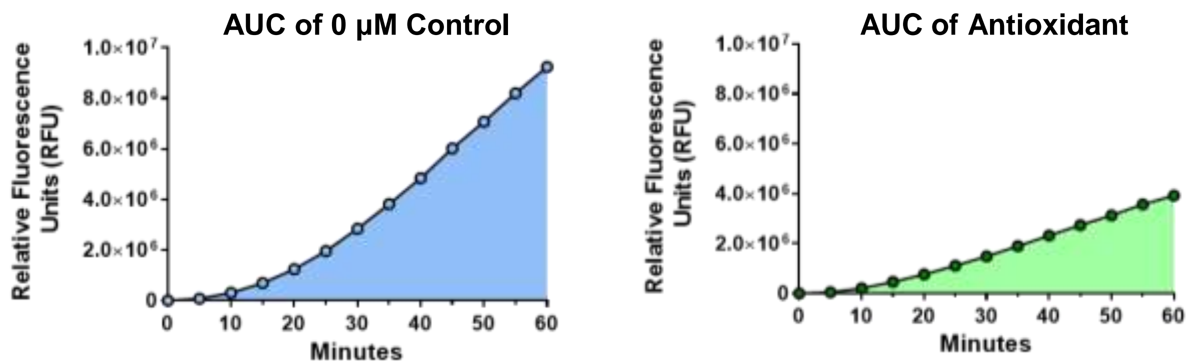


Figure 2. Illustration of AUC in an example control and antioxidant samples.
After kinetic reading is complete, AUC can be calculated from the above formula or using software packages.

2. Use following formula and the AUC values to determine the Cellular Antioxidant Activity (CAA) units:

$$\text{CAA Units} = 100 - ((AUC_{Antioxidant}/AUC_{Control}) \times 100)$$

3. Generate a dose-response curve by plotting CAA units versus Quercetin concentration. Calculate the Quercetin Equivalents (QE) value on unknown samples using the Quercetin antioxidant standard curve.

QUERCETIN STANDARD CURVE ___________________

Generate standard curve: see example below from data collected using a SpectraMax iD3.
Prepare a new standard curve for each assay performed.
[DO NOT use this standard curve to generate your data. This is an example.]

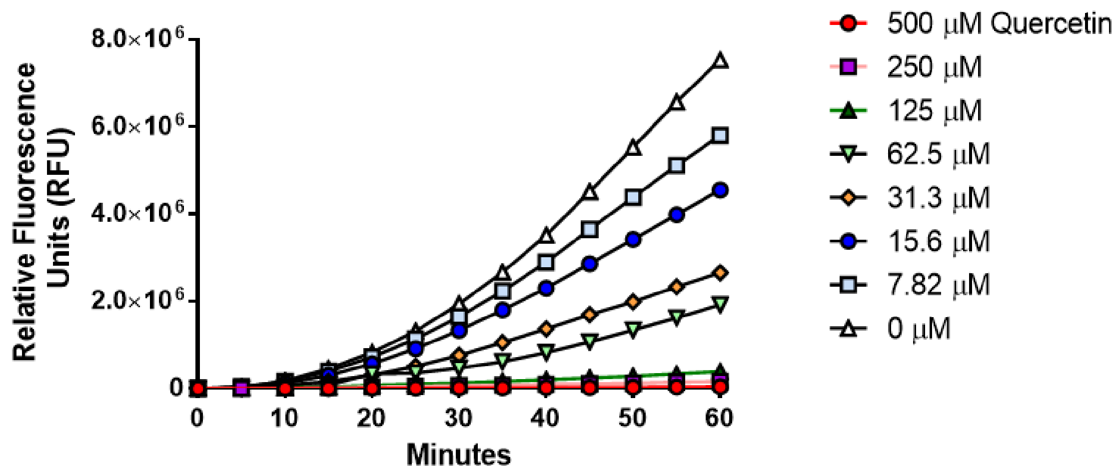


Figure 3. Cellular antioxidant activity of quercetin in adherent HeLa Cells.
20,000 HeLa cells were seeded and cultured in a black, clear bottom TC-treated 96-well plate until confluent. Afterwards, cells were pretreated with DCFH-DA and a 7-point, 2-fold dilution of quercetin starting at 500 µM for 1 hour at 37°C. Next, Radical Initiator was added to the cells, and fluorescence of DCF was captured every 5 minutes for 60 minutes at 37°C.

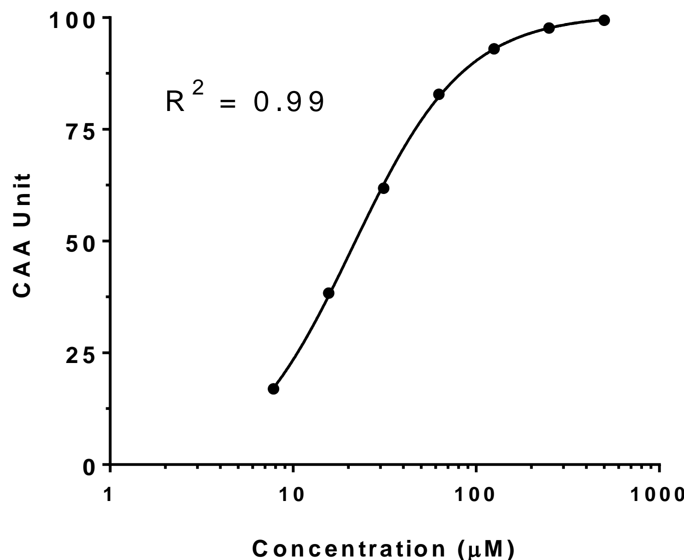
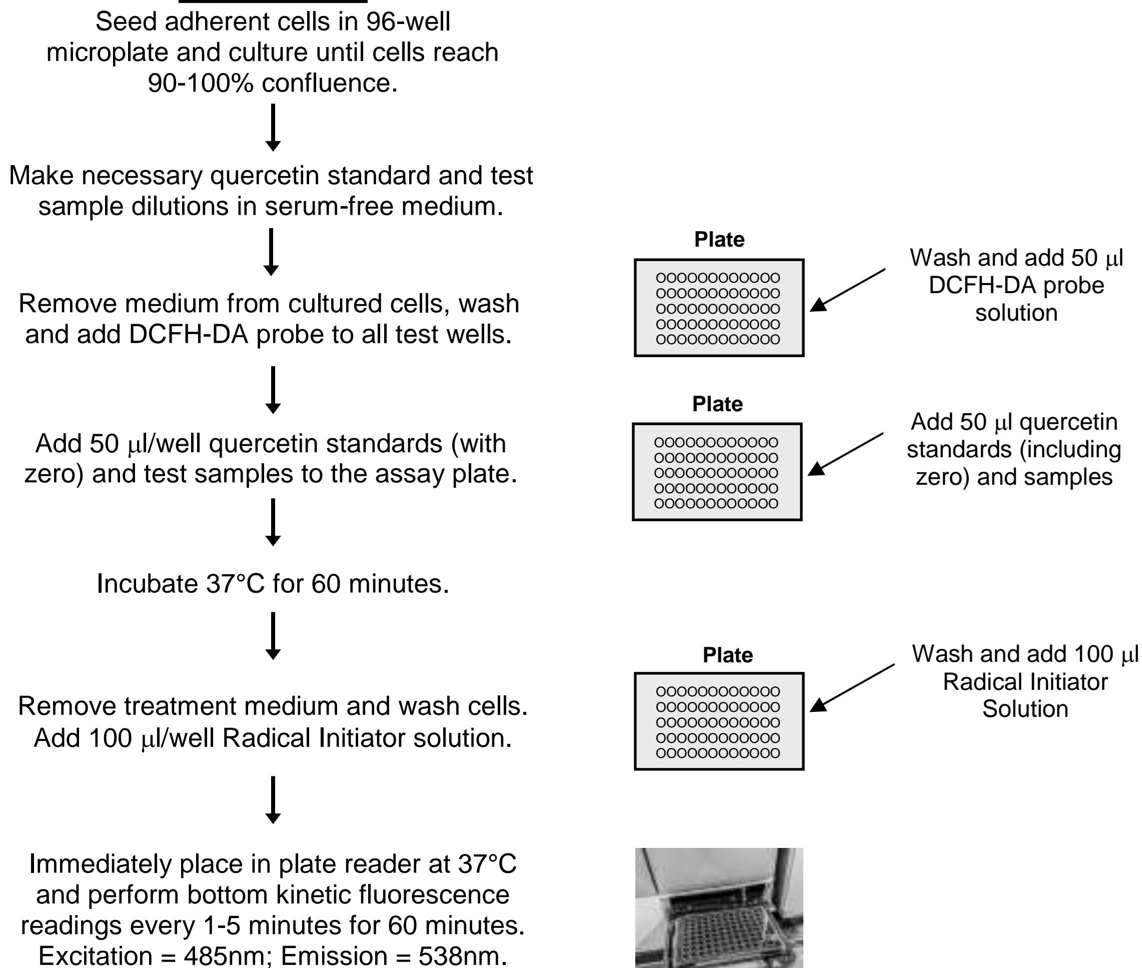


Figure 4. Dose-response curve of quercetin standard.
The area under of the curve (AUC) was calculated for each concentration of quercetin and vehicle control (0 µM quercetin). The CAA Unit was calculated from the AUCs, and CAA unit was plotted versus quercetin concentration. A 4-point logistic regression (R^2 =0.99) was generated using GraphPad Prism.

APPENDIX A: 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



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1. *J. Agric. Food Chem.* **55**:8896-8907, 2007.
2. *Food Chem.* **244**:359-363, 2018.
3. *J. Agric. Food Chem.* **56**: 8404-8411, 2008

FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my fluorescence values are different than those in the sample data but still generate a robust Quercetin standard curve?** Yes, the fluorescence values detected by the fluorimeter are based on the sensitivity of the instrument used. Our data was collected using a SpectrMax iD3 multi-mode microplate reader, and other instruments vary in sensitivity and can give lower relative fluorescence values. If the Quercetin standards still generate a robust standard curve, the assay is functioning appropriately.

2. **Should I dilute my sample for testing its CAA activity?** In order to accurately determine the CAA units of your sample, the CAA value must fall on the Quercetin standard curve. We recommend preparing several serial dilutions of your test sample in serum-free medium to ensure that you generate usable Fluorescence and AUC values.

3. **Will different cell types and percent confluence generate different CAA values?** Yes. The amount of intracellular ROS generated and the capability of antioxidants to accumulate intracellularly is dependent on both cell type and cell number. We suggest investigating the appropriate cell type and culture conditions prior to performing the assay.

4. **What concentrations of Quercetin should I use for my cell type?** Quercetin activity is dependent on cell type, such as HeLa or HepG2 cells. The suggested concentrations on page 6 work well for HepG2 cells, whereas the concentrations in the example on page 9 work well for HeLa cells. Other cell types may vary, however, the range is typically between 2.5 – 250 µM.