KAMIYA BIOMEDICAL COMPANY

Cellular Antioxidant Activity Assay

For measuring antioxidant activity within adherent cells

Cat. No. KT-931

For Research Use Only. Not for use in diagnostic procedures.

Product Information

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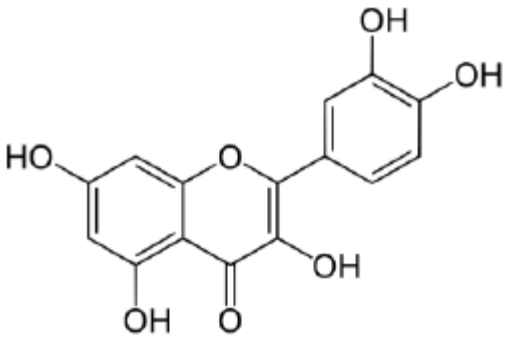
INTRODUCTION

Accumulation of reactive oxygen species (ROS) coupled with an increase in oxidative stress has been implicated in the pathogenesis of several disease states. The role of oxidative stress in vascular diseases, diabetes, renal ischemia, atherosclerosis, pulmonary pathological states, inflammatory diseases, and cancer has been well established. Free radicals and other reactive species are constantly generated *in vivo* and cause oxidative damage to biomolecules, a process held in check by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lipids. Measuring the effect of antioxidant therapies, phytochemical screening, and dietary monitoring of ROS activity within cells is crucial to suppressing or treating oxidative stress inducers.

Although many antioxidant activity assays exist, most do not measure *in vivo* activity accurately because they do not account for physiological conditions such as pH, temperature, or antioxidant bioavailability. The Cellular Antioxidant Assay Kit (Green Fluorescence) is a cell-based assay for measuring antioxidant activity within a cell in a standard cell culture environment. This environment accounts for the temperature, pH, uptake, metabolism and efficacy of antioxidants in a whole cell. The assay employs the cell-permeable fluorogenic probe dye 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA). In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent 2', 7'-Dichlorodihydrofluorescin (DCFH), which is rapidly oxidized by free radicals to highly fluorescent 2', 7'-Dichlorodihydrofluorescein (DCF) (Figure 1). The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The effect of antioxidant compounds on DCF formation can be measured against the fluorescence inhibition of the Quercetin calibrator. Quercetin is a plant flavonoid widely found in fruits and vegetables. The kit has a Quercetin detection sensitivity limit of approximately 10 µM. Each kit provides sufficient reagents to perform up to 192 assays, including calibration curve and unknown samples.



Quercetin

PRINCIPLE

The Cellular Antioxidant Assay Kit is a cell-based assay for measuring antioxidant activity within adherent cells. Cells are first cultured in a 96-well black fluorescence cell culture plate until confluent. Then the cells are pre-incubated with a cell-permeable DCFH-DA fluorescence probe dye and the bioflavonoid Quercetin, or the antioxidant sample being tested. After a brief incubation, the cells are washed, and the reaction started by adding the Free Radical Initiator. The Free Radical Initiator creates free radicals that convert the probe to highly fluorescent DCF. The Quercetin inhibits the formation of free radicals, and thus DCF formation, in a concentration dependent manner.

Fluorescence is measured over time in a standard microplate fluorometer. This fluorescence correlates to the Quercetin's ability to quench free radicals. Test antioxidant values can be compared to Quercetin to determine antioxidant activity within the cell.

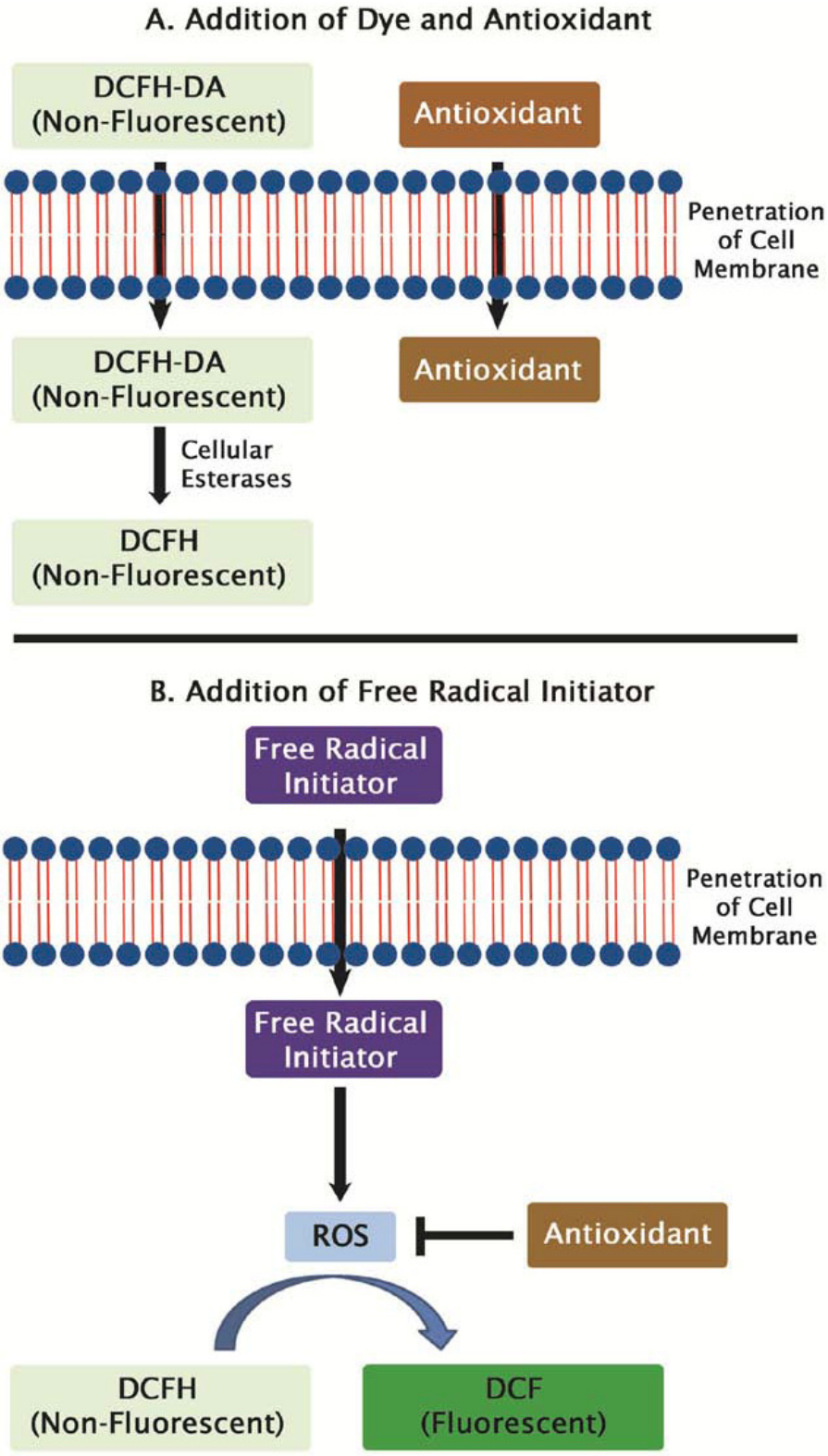


Figure 1. Mechanism of Cellular Antioxidant Assay

COMPONENTS

1. 96-well Cell Culture Microtiter Plate: Two 96-well tissue culture treated clear bottom black plates.
2. DCFH-DA Probe (1,000X): One 100 µL amber tube of a 1,000X solution in methanol.
3. Free Radical Initiator: One 200 mg tube of powder.
4. Quercetin: One 250 µL tube of a 50 mM solution in DMSO.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Adherent cell lines and test samples
2. Sterile DPBS or HBSS for washes and component dilutions
3. 10 µL to 1,000 µL adjustable single channel micropipettes with disposable tips
4. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
5. Multichannel micropipette reservoir
6. Cell culture incubator (37ºC, 5% CO_2 atmosphere)
7. Cell culture medium (ie: DMEM +/-10% FBS)
8. Fluorescent microplate reader capable of reading 480 nm (excitation) and 530 nm (emission)

STORAGE

Upon receipt, store the DCFH-DA Probe (1,000X) and Quercetin at -20ºC. Avoid multiple freeze/thaw cycles. Store the remaining components at 4ºC.

REAGENT PREPARATION

• 2X DCFH-DA Probe: Dilute the DCFH-DA Probe (1,000X) stock solution to 2X in cell culture media, preferably without FBS. Stir or vortex to homogeneity. Label this as 2X DCFH-DA Probe/Media Solution. Prepare only enough for immediate applications.
Note: Due to light-induced auto-oxidation, DCFH-DA fluorescence probe solutions at any concentration must be protected from light.
• Free Radical Initiator: Freshly weigh out and prepare a 2.8% solution of the Free Radical Initiator in 1X sterile DPBS. (eg. Reconstitute 28 mg of powder in 1,000 µL DPBS). This is a 100X solution. Prior to use, dilute the Free Radical Initiator 1:100 in DPBS. Vortex thoroughly. The 100X Free Radical Initiator solution is stable for 1 week when stored at -20ºC.

SAMPLE PREPARATION

Note: Samples should be stored at -70ºC prior to performing the assay. Samples should be prepared at the discretion of the user. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

Prepare all samples by diluting and titrating in cell culture media. Samples will be diluted 1:2 final within the assay.

• Nutrition Extracts: Results may vary depending on sample source and purification. Dilution and preparation of these samples is at the discretion of the user, but use the following guidelines:

Weigh solid sample and then blend with a Waring blender for 5 min. in chilled 80% acetone (1:2 w/v, eg. 1 g sample in 2 mL of 80% acetone). Next, homogenize the samples for 3 min. Filter through Whatman paper and evaporate filtrate under vacuum. Reconstitute samples in 70% methanol and store at -80ºC. Before use, evaporate methanol, preferably under nitrogen, and reconstitute extracts with deionized water or DPBS. Samples may also be diluted in cell culture medium. Final treatment solutions should contain 2% or less of solvent to prevent cytotoxicity.

CALIBRATOR PREPARATION

Immediately before use, prepare a 1:25 dilution of the stock Quercetin in cell culture media. Use this Quercetin solution to prepare a series of Quercetin calibrators in the concentration range of 0 µM – 2,000 µM in cell culture media according to the table below (see Table 1).

Calibrator Tubes	Quercetin Calibrator (µL)	Cell Culture Media (µL)	Quercetin (µM)
1	40	960	2,000
2	500 of Tube #1	500	1,000
3	500 of Tube #2	500	500
4	500 of Tube #3	500	250
5	500 of Tube #4	500	125
6	500 of Tube #5	500	62.5
7	500 of Tube #6	500	31.3
8	0	1,000	0

Table 1. Preparation of Quercetin Calibrators

ASSAY PROCEDURE

Note: Each Antioxidant Calibrator and sample should be assayed in duplicate or triplicate. A freshly prepared calibration curve should be used each time the assay is performed.

1. Culture cells such as HepG2 or HeLa in the 96-well Cell Culture Microtiter Plate until cells are 90% to 100% confluent.
2. Carefully remove media from all wells and discard. Wash cells gently 3 times with DPBS or HBSS. Remove the last wash and discard.
3. Add 50 µL of DCFH-DA Probe solutions to all wells with confluent cells to be tested.
4. Add 50 µL of Quercetin Calibrator or prepared sample to each well with confluent cells to be tested. Incubate at 37ºC for 60 minutes.
5. Carefully remove the solution. Wash 3 times with DPBS or HBSS. Remove the last wash and discard.
6. Add 100 µL of the Free Radical Initiator solution to all wells. Immediately begin reading wells with a fluorescent microplate reader at 37ºC with an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Read the wells in increments between 1 and 5 minutes for a total of 60 minutes. Save values for Calculation of Results.

EXAMPLE OF RESULTS

The following figures demonstrate typical Intracellular Antioxidant Assay results. Fluorescence measurement was performed on SpectraMax Gemini XS Fluorometer (Molecular Devices) with a 485/538 nm filter set and 530 nm cutoff. One should use the data below for reference only. This data should not be used to interpret actual results.

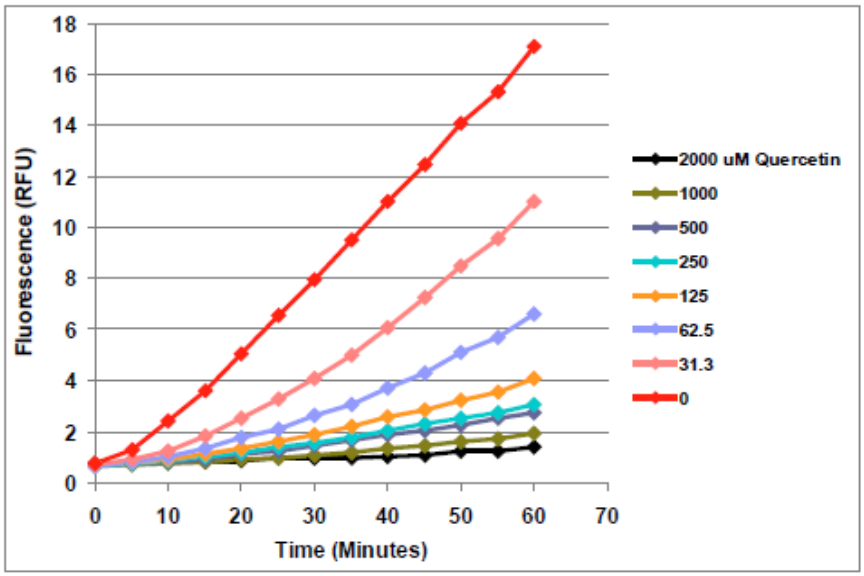


Figure 2. Cellular Antioxidant Activity of Quercetin in HeLa Cells. 60,000 HeLa cells were seeded and cultured in a 96-well plate until confluent. Cells were then pretreated with DCFH-DA and Quercetin for 60 minutes at 37ºC. Free Radical Initiator was then added to the cells to begin the assay, which was read every five minutes for 1 hour at 37ºC.

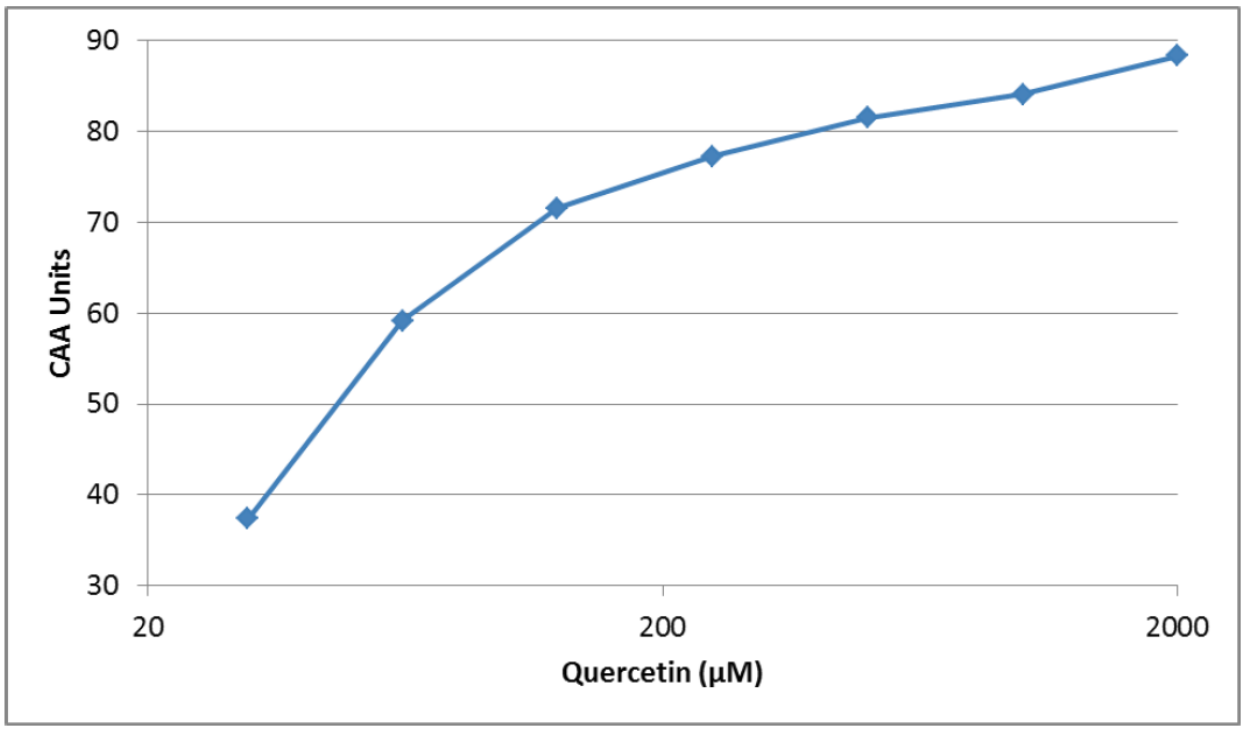


Figure 3. Dose-Response Curve of Quercetin Calibrator.

CALCULATION OF RESULTS

Note: A spreadsheet application or plate reader software can be used to perform the calculations.

1. Calculate the integrated area under the curve (AUC) for each sample and calibrator using the final assay values and the linear regression formula below:

AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + RFU_3/RFU_0 +……+ RFU_{59}/RFU_0 + RFU_{60}/RFU_0
RFU_0 = relative fluorescence value of time point zero.
RFU_x = relative fluorescence value of time points (e.g. RFU_5 is relative fluorescence value at minute five)

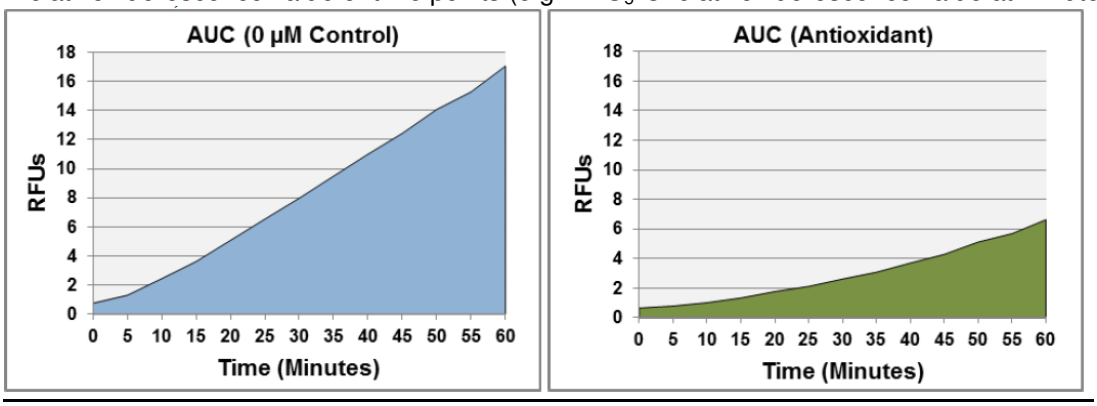


Figure 4. Example Data for Calculation of AUC for Antioxidant Sample and Negative Control.

2. Use the AUC values to determine the Cellular Antioxidant Activity values according to the formula:

CAA Units = 100 – ($AUC_{Antioxidant}$ / $AUC_{Control}$) x 100

3. Plot a dose-response curve by graphing CAA units versus Quercetin concentration (see Figure 3). Based on the Quercetin antioxidant calibration curve, determine the equivalent Quercetin Equivalents (QE) value of unknown samples.

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KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com