Fluorescent Microplate Based

Hydroxyl Radical Antioxidant Capacity [HORAC] Assay

PLEASE READ ALL INSTRUCTIONS PRIOR TO USING THIS PRODUCT.

INTRODUCTION

It is now well established that oxidative stress is a major risk factor for the development of several diseases including atherosclerosis, cardiovascular disease, and cancer. Oxidative stress is the condition in which there is an imbalance between the concentrations of reactive oxygen species (ROS) and physiological antioxidants, resulting in oxidative damage to many biomolecules within the cell. Products of ROS-mediated oxidation are widely used to monitor oxidative stress. However, it is also important to assess the antioxidant capacity of cells and biological fluids, as well as putative "functional foods" to assess their antioxidant capacity. Organisms possess multiple antioxidant systems to help regulate ROS and prevent oxidative stress. In vertebrates, these include enzymes that metabolize ROS, antioxidant proteins, and smaller molecules that are important antioxidants. These antioxidants include hydrophilic as well as lipid-soluble molecules that are localized throughout various tissues and cell types.

Classification	Examples
Enzymes:	superoxide dismutase, catalase, glutathione peroxidase
Large Molecule:	albumin, ferritin, ceruloplasmin
Small Molecule:	ascorbic acid, _-tocopherol, _-carotene, uric acid, bilirubin, glutathione, methionine

Given the multiplicity of antioxidant pathways, their centrality in the prevention of oxidative stress, and the influences of lifestyle and nutritional supplements on an individual's antioxidant capacity, it is important to be able to quantitatively measure the total antioxidant capacity or antioxidant power in a biological specimen or in nutrients.

PRINCIPLES OF PROCEDURE

The HORAC assay is based on the oxidation of fluorescein by hydroxyl radicals *via* a classic hydrogen atom transfer (HAT) mechanism. Free radicals are generated by hydrogen peroxide (H_2O_2). The hydroxyl radicals thus generated quench the fluorescence of fluorescein over time. The antioxidants block the hydroxyl radical mediated oxidation of fluorescein until all of the antioxidant activity in the sample is exhausted, after which the H_2O_2 radicals react with and quench the fluorescence of fluorescein. The area under the fluorescence decay curve (AUC) is used to quantify the total hydroxyl radical antioxidant activity in a sample and is compared to a standard curve obtained using various concentrations of gallic acid. Unlike other antioxidant activity assays, the fluorescent HORAC assay provides a direct measurement of antioxidant capacity against hydrophilic chain-breaking hydroxyl radicals.

MATERIALS PROVIDED

Components	Contents	Volume	Storage	Cat no.
Assay Buffer	Phosphate buffer for diluting samples and standards	50 mL	4°C	TA 30a
Fluorescein Solution	Concentrated fluorescein in phosphate buffer	500 µL	4°C	TA 30b
Hydroxyl Radical	Hydrogen peroxide for radical generation	4 mL	4°C	TA 30c
Fenton Agent	Metal to complete Fenton reaction	4 mL	4°C	TA 30d
Standard	Gallic acid in phosphate buffer	1.5 mL	4°C	TA 30e
Plate	Black microplate with optical bottom	1	RT	TA 30f
Troughs	Plastic trays for dispensing components	3	RT	TA 30g

MATERIALS REQUIRED BUT NOT PROVIDED

1. Fluorescence microplate reader equipped with a 488 nm excitiation filter and a 515 nm emission filter.
2. Precision pipettes with a range of 20 µL to 1000 µL.
3. Multichannel pipette with a range of 20 µL to 150 µL.

PROCEDURAL NOTES

1. The entire kit should be stored at 4°C until use and all components stored on ice during use.
2. For accuracy, it is recommended that all standards and samples be run in duplicate or triplicate.

REAGENTS PREPARATION

1. The Fluorescein Solution is provided at a 100x concentration and must be diluted with Assay Buffer prior to being used in the assay. To dilute to the working concentration, add 200 µL of the Fluorescein Solution to 19.8 mL of Assay Buffer, this should be labeled as the Fluorescein Working Solution. Store at 4°C in the dark for up to one month.

STANDARD PREPARATION

In order to create a standard curve for the assay, the stock solution of the Standard must be diluted with the Assay Buffer according the layout in the table below. Once each standard tube has been created and mixed, 20 µL of each can be added to the appropriate wells on the plate.

Preparation of Standard Curve.

Standard Tube	Final Concentration (µM)	Assay Buffer (µL)	5mM Stock (µL)
B_0	0	1000	0
S_1	200	960	40
S_2	300	940	60
S_3	400	920	80
S_4	500	900	100
S_5	600	880	120
S_6	700	860	140
S_7	800	840	160

SAMPLE PREPARATION

All samples should be stored at -70°C at all times prior to assay. Apply the samples to the assay plate immediately upon thawing. Sample preparation is subject to the discretion of the user and deviations of the following recommendations may be implemented to optimize or better complement experimental design.

Deproteinated Fractions: All of the following samples can be deproteinated and have their non-protein fractions assayed. To measure the HORAC value in the non-protein fraction, mix the sample with 0.5 M perchloric acid (PCA) (1:1, v/v) or acetone (1:4, v/v), centrifuge at 10,000 x g for 10 minutes at 4°C, and recover the supernatant for the assay.

Lipophilic Fractions: Antioxidants fall into two classes, hydrophilic and lipophillic, with the later requiring enhancement of solubility in order to measure their capacity in an aqueous environment. One method that can be employed is to dissolve a sample in 100% acetone and then take a portion and dilute it into a solution of 7% β-cyclodextrin and 50% acetone. Incubate the sample for 1 hour at room temperature with shaking and then further dilute before running in the assay.

Tissue Lysate: Homogenize the tissue sample on ice with ice cold PBS then centrifuge at 10,000 x g for 10 minutes at 4°C. Aliquot the supernatant for use in protein determination and running the assay.

Cell Culture: Wash the cells 2-3 times with ice cold PBS prior to lysis. Lyse cells by homogenization or sonication with ice cold PBS then centrifuge at 10,000 x g for 10 minutes at 4°C. Aliquot the supernatant for use in protein determination and running the assay.

Urine: Collected samples may be assayed directly or diluted with Assay Buffer where appropriate.

Plasma: Collect the blood with heparin and centrifuge at 4°C for 10 minutes. Remove the plasma and aliquot for use in protein determination and running the assay. Blood plasma or serum needs to be diluted 100- to 200-fold with Assay Buffer before it is used in the assay.

Nutrition Science: The assay may vary considerably among nutritional substances. Dilution and preparation of nutritional science samples will require foresight and discretion on the part of the user. In many cases, aqueous based samples such as juice and tea can be assayed directly without further processing. Solid food samples or aqueous based samples that contain high amounts of protein and/or fiber may be processed in the following manner:

> **Solid:** Weigh the solid and then homogenize with a blender after adding deionized water (e.g., 1:2, w/v). The homogenate is then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant (water-soluble fraction) is recovered. The water-insoluble fraction (pulp) is washed with deionized water, and the recovered supernatant is pooled with the supernatant obtained from the first centrifugation step. The pooled supernatant is used directly for the assay after suitable dilution with Assay Buffer. The pulp is then further extracted by using pure acetone [1:4, w(pulp)/v] with shaking at room temperature for 30 minutes. The acetone extract is recovered after centrifugation at 12,000 x g for 10 minutes at 4°C and is used for the assay after suitable dilution with Assay Buffer. The HORAC activity of the sample is then calculated by adding the HORAC activity from its water-soluble fraction and its acetone-extracted pulp fraction.
> **Aqueous:** Centrifuge at 5,000 x g for 10 minutes at 4°C to remove particulates then dilute supernatant as needed to run in the assay.

PROCEDURE

1. Place 20 µL of Sample or Standard into the appropriate wells of the provided microplate. See the Suggested Plate Layout on the next page for a potential organization of the assay.
2. Place 140 µL of the Fluorescein Working Solution into each of the wells.
3. Incubate the plate in the fluorescent plate reader for 30 minutes at 37°C. It is suggested that a sample reading be taken at the beginning and end of this incubation at an excitation wavelength of 488 nm and an emission wavelength of 515 nm.
4. Place 20 µL of the Hydroxyl Radical into each well using either a multichannel pipette or built-in dispensing unit of the fluorescent plate reader.
5. Place 20 µL of the Fenton Reagent into each well using either a multichannel pipette or built-in dispensing unit of the fluorescent plate reader.
6. Shake the plate at 1200 rpm for 10 seconds in the fluorescence plate reader to mix all of the components.
7. Immediately start reading samples with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. Samples should be read every minute for a total of 45 minutes.

Note: Final readings must be less than 10 percent of the initial readings in order for the assay to be run to completion. If final sample readings are not less than 10 percent of the initial readings, further sample dilution is necessary, and the assay must be repeated.

CALCULATIONS

1. Calculate the area under the curve (AUC) for each of the Samples and Standards using the formula below and the reading collected in Step 6 of the assay.
2. Calculate the Net AUC by subtracting the Blank (B_0) AUC from the AUC of each Sample and Standard.
3. Plot the Net AUC versus the concentration of Gallic Acid. See the Sample Standard Curve below.
4. Determine the µM Gallic Acid Equivalents (GAE) by using the formula generated to create the standard curve. Sample results can be expressed as either GAE per mL of sample, GAE per mg of protein or GAE per g of sample.

The AUC should be calculated using the plate reader software or in a spreadsheet program using the following formula:

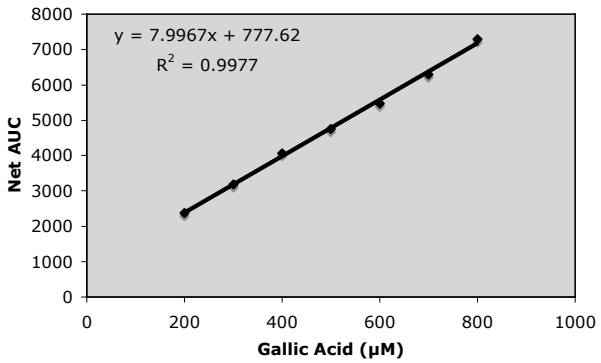
$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_{59}/f_0 + f_{60}/f_0$$

Gallic Acid Equivalents are calculated using the line equation from the standard curve using the following designations:

$Y = mX + b$ or $X = (Y - b) / m$ where:

X = Gallic Acid Equivalents
Y = The Net AUC
m = The slope
b = The Y-intercept

Sample Standard Curve



Note: This is a sample standard curve and should not be used to determine the HORAC value of samples.

Suggested Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}

B_0 is the blank, S_1-S_7 are standards, and U_1 – U_{40} are unknown samples.

REFERENCES

1. Ou, B., Hampsch-Woddill, M., Flanagan, J., Deemer, EK., Prior, RL., Huang, D. *J Agric Food Chem.* **50,** 2772 (2002).

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