Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay Kit (Fluorometric)

Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay Kit (Fluorometric) can be used to measure HORAC activity in serum, plasma, urine, tissue lysate and cell culture supernatants.

Catalog number: ARG82222

Package: 2 X 96 assays

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

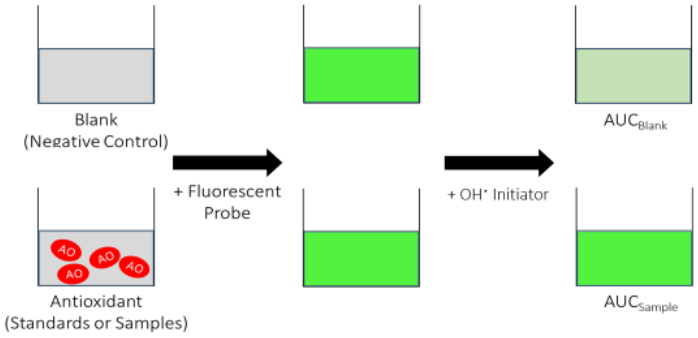
INTRODUCTION

The Folin–Ciocâlteu reagent (FCR) or Folin's phenol reagent or Folin–Denis reagent, also called the gallic acid equivalence method (GAE), is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric in vitro assay of phenolic and polyphenolic antioxidants

The reagent does not measure only phenols, but will react with any reducing substance. It therefore measures the total reducing capacity of a sample, not just phenolic compounds. This reagent is part of the Lowry protein assay, and will also react with some nitrogen-containing compounds such as hydroxylamine and guanidine. The reagent has also been shown to be reactive towards thiols, many vitamins, the nucleotide base guanine, the trioses glyceraldehyde and dihydroxyacetone, and some inorganic ions. Copper complexation increases the reactivity of phenols towards this reagent. [Provide by Wikipedia: Gallic Acid Equivalence method]

PRINCIPLE OF THE ASSAY

The Hydroxyl Radical Antioxidant Capacity (HORAC) assay is based on the oxidation of fluorescein by hydroxyl radicals via a classic hydrogen atom transfer (HAT) mechanism. Hydroxyl radicals are produced by hydroxyl radical initiator and fenton reagent, which quenches the fluorescent probe over time. Antioxidants present in the assay work to block the radical hydroxyl oxidation of the fluorescent probe until the antioxidant activity in the sample is depleted. The remaining hydroxyl radicals destroy the fluorescence of the fluorescent probe. This assay continues until completion, which means both the antioxidant's inhibition time and inhibition percentage of free radical damage is a single value. The sample antioxidant capacity correlates to the fluorescence decay curve, which is usually represented as the area under the curve (AUC). The AUC is used to quantify the total hydroxyl radical antioxidant activity in a sample and is compared to a gallic acid antioxidant standard curve.



Integration: Net AUC (HORAC Capacity) = $AUC_{Sample} - AUC_{Blank}$

MATERIALS PROVIDED & STORAGE INFORMATION

Upon receipt, store the 100X Fluorescein Probe and Antioxidant Standard at -20°C. Aliquot as necessary to avoid multiple freeze/thaw cycles. Store all remaining kit components at 4°C. Use the kit before expiration date.

Component	Quantity	Storage information
96-Well Fluorescence Microplate	2 X 96	4°C
100X Fluorescein Probe	0.5 mL	-20°C
5X Hydroxyl Radical Initiator	1 mL (amber tube)	4°C
Antioxidant Standard (5 mM Gallic Acid)	1.5 mL	-20°C
Fenton Reagent	5 mL	4°C
2X Assay Diluent	50 mL	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescence microplate reader equipped with a 480 nm excitation filter and 530 nm emission filter
- 37°C Incubator
- Deionized or Distilled water
- Reagents and materials necessary for sample extraction and purification
- Pipettes and pipette tips
- Multichannel micropipette reservoir

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon receipt, store the 100X Fluorescein Probe and Antioxidant Standard at -20°C. Aliquot as necessary to avoid multiple freeze/thaw cycles. Store all remaining kit components at 4°C.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended assaying the Standards and samples in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

Samples should be stored at-70ºC prior to performing the assay. Sample 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.

Deproteinated Fractions: Samples can be deproteinated and have their non-protein fractions assayed. Mix samples with 0.5 M perchloric acid (1:2, v/v), centrifuge at 10,000 x g for 10 minutes at 4°C. Remove the supernatant for measuring the non-protein fraction in the assay.

Cell Culture: Wash cells 3 times with 1X cold PBS prior to lysis. Lyse cells with sonication or homogenation in 1X cold PBS and centrifuge at 10,000 x g for 10 minutes at 4°C. Aliquot and store the supernatant for use in the assay.

Lipophilic Fractions: Lipophilic samples must be treated in order to be soluble in an aqueous environment. Dissolve samples in 100% acetone and then dilute in a solution of 7% beta-cyclodextrin and 50% acetone. Incubate the mixture for 1 hour at room temperature with mixing. Further dilute samples as necessary prior to testing.

Plasma: Collect blood with EDTA, heparin or citrate and centrifuge at 2000 x g and 4ºC for 10 minutes. Sample should be tested immediately or frozen at -70°C for storage.

Serum: Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Sample should be tested immediately or frozen at -70°C for storage.

Tissue Lysate: Sonicate or homogenize tissue sample on 1X cold PBS and centrifuge at 10,000 x g for 10 minutes at 4°C. Aliquot and store the

supernatant for use in the assay.

Urine: Test neat or diluted with Assay Diluent if appropriate.

Nutrition Samples: 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:

A. **Solid or High Protein Samples:** weigh solid sample and then homogenize after adding deionized water (1:2, w/v). Centrifuge the homogenate at 12000 x g for 10 minutes at 4°C. Recover the supernatant which is the water-soluble fraction. Separately recover the insoluble fraction (pulp) and wash with deionized water. Combine this wash with the supernatant. The pooled supernatant can be diluted with Assay Diluent and used directly in the assay. The pulp is further extracted by adding pure acetone (1:4, w/v) and mixing at room temperature for 30-60 minutes. Centrifuge the extract / solid at 12,000 x g for 10 minutes at 4°C. Recover the acetone extract and dilute with Assay Diluent as necessary prior to the assay. The total ORAC value is calculated by combining the result from the water-soluble fraction and the acetone extract from the pulp fraction.

B. **Aqueous Samples:** Centrifuge the sample at 10,000 x g for 10 minutes at 4°C to remove any particulates. Dilute the supernatant as necessary prior to running the assay. Certain liquids such as juice extracts may be tested without dilution.

REAGENT PREPARATION

- 1X Assay Diluent: Dilute the 2X Assay Diluent into deionized water to yield 1X Assay Diluent. (E.g., add 20 mL of 2X Assay Diluent into 20 mL deionized water to a final volume of 40 mL) Store the 1X Assay Diluent at 4°C.
- 1X Fluorescein Probe: Dilute the 100X Fluorescein Probe into 1X Assay Diluent to yield 1X Fluorescein Probe. (E.g., add 0.1 mL of 100X Fluorescein Probe into 9.9 mL 1X Assay Diluent to a final volume of 10 mL) Use only enough Fluorescein Probe as necessary for immediate applications. Do not store diluted Fluorescein Probe solution.
- 1X Hydroxyl Radical Initiator Solution: Dilute the 5X Hydroxyl Radical Initiator into deionized water to yield 1X Hydroxyl Radical Initiator. (E.g., add 0.1 mL of 5X Hydroxyl Radical Initiator into 0.4 mL of deionized water to a final volume of 0.5 mL) Mix to homogeneity. Label this solution as 1X Hydroxyl Radical Initiator Solution. Use only enough Hydroxyl Radical Initiator as necessary for immediate use.

Note: Do not store Fluorescein Probe or Hydroxyl Radical Initiator Solution.

- Standards: Prepare fresh standards by diluting the 5 mM Gallic Acid Antioxidant Standard stock solution in 1X Assay Diluent. Use only enough Gallic Acid Antioxidant Standard as necessary for immediate applications. Prepare a serial of the remaining antioxidant standards according to the table below.

Standard tube	Final Gallic Acid conc. (µM)	1X Assay Diluent (µL)	Volume of 5 mM antioxidant Standard (µL)
S1	900	410	90
S2	800	420	80
S3	700	430	70
S4	600	440	60
S5	500	450	50
S6	400	460	40
S7	300	470	30
S8	200	480	20
S9	100	490	10
S0	0	500	0

Note: Do not store diluted Antioxidant Standard solutions. Prepare fresh Antioxidant Standards for every assay performed.

ASSAY PROCEDURE

Standards and samples should be assayed in duplicates or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add 20 µL of serial diluted Standards or samples into 96-well fluorescence microplate.
2. Add 140 µL of 1X Fluorescein Probe to each well. Mix thoroughly.
3. Incubate at room temperature for 30 minutes.
4. Add 20 µL of the 1X Hydroxyl Radical Initiator Solution into each well.
5. Immediately add 20 µL of the Fenton Reagent to each well. Shake the plate immediately for 15 seconds to ensure homogeneity.
6. Read the plate with a fluorescence microplate reader at 37°C with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.
7. Read the wells in increments between 1 and 5 minutes for a total of 60 minutes. Save values for Calculation of Results below.

Note: The final assay values of blank control should be less than 10% of the initial values in order for the assay to be completed.

CALCULATION OF RESULTS

A plate reader software can be used to perform the calculations.

1. Calculate the area under the curve (AUC) for each sample and standard using the final assay values and the linear regression formula below. The AUC can be calculated from the equation below:

$AUC = 1 + RFU_1/RFU_0 + RFU_2/RFU_0 + RFU_3/RFU_0 + \ldots\ldots + RFU_{59}/RFU_0 + RFU_{60}/RFU_0$

RFU_0 = relative fluorescence value of time point zero.

RFU_X = relative fluorescence value of time points X (E.g., RFU_5 is relative fluorescence value at minute 5)

2. Calculate the Net AUC by subtracting the Blank AUC from the AUC of each sample and standard.

Net AUC = AUC (Antioxidant) – AUC (blank)

3. Graph the Net AUC on the y-axis against the Gallic Acid Antioxidant Standard concentration on the x-axis.
4. Calculate the µMole Gallic Acid Equivalents (GAE) of samples by comparing the standard curve. Results (HORAC value) may be reported as GAE/L.

Calculation Example:

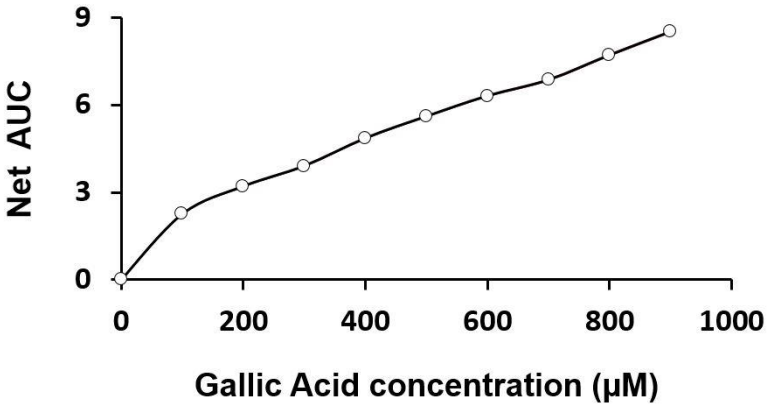
20 µL of 10-fold diluted sample is assayed along with 20 µL of each Gallic Acid antioxidant standard including blank as described in Assay Protocol. The average AUC is 2.2 for blank and 7.0 for sample.

Net AUC = 7.0 – 2.2 = 4.8

Based on the Gallic Acid antioxidant standard curve, the equivalent Gallic concentration is 20 µM, therefore: ORAC value (Sample) = 400 µM x 10 (dilution factor) = 4000 µM GAE = 4000 µMole GAE/L

EXAMPLE OF TYPICAL STANDARD CURVE

The following figures demonstrate typical with the Hydroxyl Radical Antioxidant Capacity (HORAC) Activity Assay kit. One should use the data below for reference only. This data should not be used to interpret actual results.



QUALITY ASSURANCE

Intra-assay and Inter-assay precision

The CV value of intra-assay and inter-assay was ≤ 10 %