Oxygen Radical Antioxidant Capacity (ORAC) Assay Kit

Oxygen Radical Antioxidant Capacity (ORAC) Assay Kit is a detection kit for the quantification of ORAC in serum, plasma, urine, tissue lysate, lipophilic fractions, deproteinated fractions and cell culture supernatants.

Catalog number: ARG82221

Package: 2 X 96 assays

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: 9F.-7, No. 12, Taiyuan 2nd St., Zhubei City,

Hsinchu County 302082, Taiwan

Phone: +886 (3) 621 8100

Fax: +886 (3) 553 0266

Email: info@arigobio.com

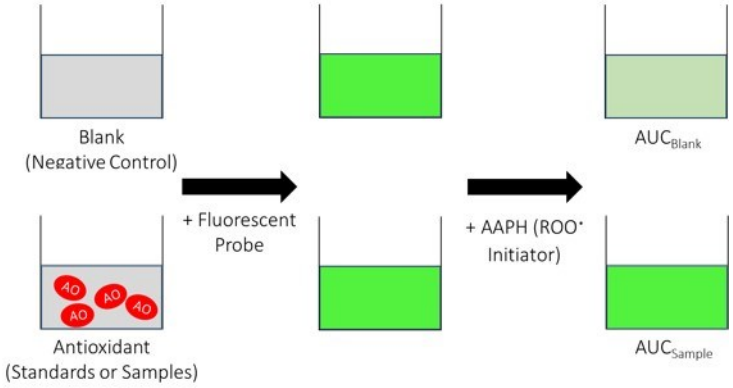
INTRODUCTION

Oxygen radical antioxidant capacity (ORAC) was a method of measuring antioxidant capacities in biological samples *in vitro.* Because no physiological proof *in vivo* existed in support of the free-radical theory or that ORAC provided information relevant to biological antioxidant potential, it was withdrawn in 2012.

Various foods were tested using this method, with certain spices, berries and legumes rated highly in extensive tables once published by the United States Department of Agriculture (USDA). Alternative measurements include the Folin-Ciocalteu reagent, and the Trolox equivalent antioxidant capacity assay. [Provide by Wikipedia: Oxygen radical antioxidant capacity]

PRINCIPLE OF THE ASSAY

The Oxygen Radical Antioxidant Capacity (ORAC) Assay is based on the oxidation of a fluorescent probe by peroxyl radicals by way of a hydrogen atom transfer (HAT) process. Peroxyl radicals are produced by a free radical initiator, which quenches the fluorescent probe over time. Antioxidants present in the assay work to block the peroxyl radical oxidation of the fluorescent probe until the antioxidant activity in the sample is depleted. The remaining peroxyl 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 peroxyl radical antioxidant activity in a sample and is compared to an antioxidant standard curve. (See Assay Principle below).



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

MATERIALS PROVIDED & STORAGE INFORMATION

Upon receipt store the Fluorescent Probe and Antioxidant Standard frozen at -20°C. Aliquot as necessary to avoid multiple freeze/thaws. Store all remaining 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
Free Radical Initiator	0.5 g	4°C
Antioxidant Standard (5 mM Trolox solution)	100 µL	-20°C
4X Assay Diluent	50 mL	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescence microplate reader equipped with a 480 nm excitation filter and 520 nm emission filter
- 1X PBS and Deionized water
- 50% Acetone
- 37°C incubator
- 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 Fluorescent Probe and Antioxidant Standard frozen at -20°C. Aliquot as necessary to avoid multiple freeze/thaws. Store all remaining components at 4°C. Components should be thawed / maintained at room temperature during assay preparation. Any unused material should be aliquoted and frozen at -80°C to avoid multiple freeze / thaws.
- The concentrations can vary over a wide range depending on the different samples. For unknown samples, we recommend doing a pilot experiment & testing several doses to ensure the readings are within the standard curve range.
- 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 -70°C prior to performing the assay. 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: Dissolve lipophilic samples in 100% acetone and then dilute in 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 4°C 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 4X Assay Diluent into deionized water to yield 1X Assay Diluent. (E.g., add 10 mL of 4X Assay Diluent into 30 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.
- Free Radical Initiator Solution: Freshly prepare 80 mg/mL Free Radical Initiator Solution in 1X PBS. E.g., weigh out 160 mg of Free Radical Initiator powder in a conical tube and reconstitute the powder with 2 mL of 1X PBS and mix to homogeneity. Free Radical Initiator Solution is not stable and should be used immediately.

- **Standards:**

➢ **Hydrophilic (aqueous) Samples:** Prepare fresh standards by diluting the 5 mM Antioxidant Standard stock solution to 0.2 mM in 1X Assay Diluent (E.g., add 10 µL of Antioxidant Standard stock into 240 µL of 1X Assay Diluent). Prepare a serial of standards according to the table below.

Standard tube	Final antioxidant (Trolox) conc. (µM)	1X Assay Diluent (µL)	Volume of 0.2 mM antioxidant Standard (µL)
S1	50	150	50
S2	40	160	40
S3	30	170	30
S4	20	180	20
S5	10	190	10
S6	5	195	5
S7	2.5	197.5	2.5
S0	0	200	0

➢ **Lipophilic Samples**: Prepare fresh standards by diluting the 5 mM Antioxidant Standard stock solution to 0.2 mM in 50% acetone (E.g., add 10 µL of Antioxidant Standard stock into 240 µL of 50% acetone). Prepare a serial of standards according to the table below.

Standard tube	Final antioxidant (Trolox) conc. (µM)	50% Acetone (µL)	Volume of 0.2 mM antioxidant Standard (µL)
S1	50	150	50
S2	40	160	40
S3	30	170	30
S4	20	180	20
S5	10	190	10
S6	5	195	5
S7	2.5	197.5	2.5
S0	0	200	0

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 25 µL of serial diluted Standards or samples into 96-well fluorescence microplate.
2. Add 150 µL of 1X Fluorescein Probe to each well. Mix thoroughly.
3. Incubate at 37°C for 30 minutes.
4. Add 25 µL of the Free Radical Initiator Solution into each well.
5. Mix the reaction mixture thoroughly by pipetting 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 520 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 Trolox™ Antioxidant Standard concentration on the x-axis.
4. Calculate the µMole Trolox Equivalents (TE) of samples by comparing the standard curve. Results (ORAC value) may be reported as TE/L.

Calculation Example:

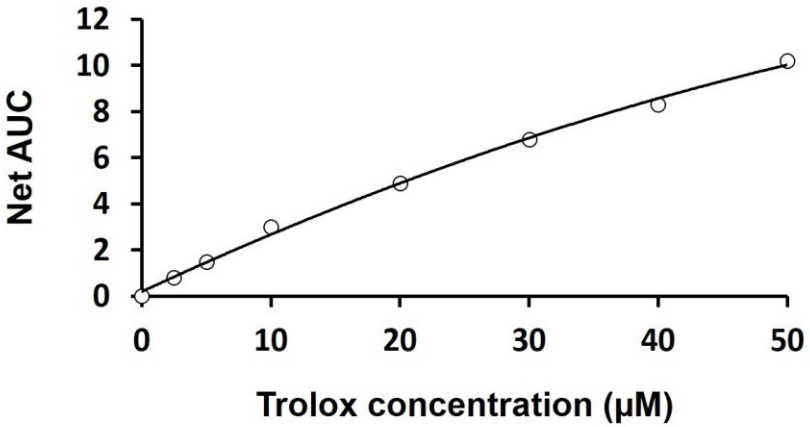
25 µL of 10-fold diluted sample is assayed along with 25 µL of each Trolox antioxidant standard including blank as described in Assay Protocol. The average AUC is 4.3 for blank and 9.1 for sample.

Net AUC = 9.1 – 4.3 = 4.8

Based on the Trolox antioxidant standard curve, the equivalent Trolox concentration is 20 µM, therefore: ORAC value (Sample) = 20 µM x 10 (dilution factor) = 200 µM TE = 200 µMole TE/L

EXAMPLE OF TYPICAL STANDARD CURVE

The following figures demonstrate typical results with Oxygen Radical Antioxidant Capacity (ORAC) 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 %