Product Manual

OxiSelect™ Oxygen Radical Antioxidant Capacity (ORAC) Activity Assay

Catalog Number

STA-345	192 assays
STA-345-5	5 x 192 assays

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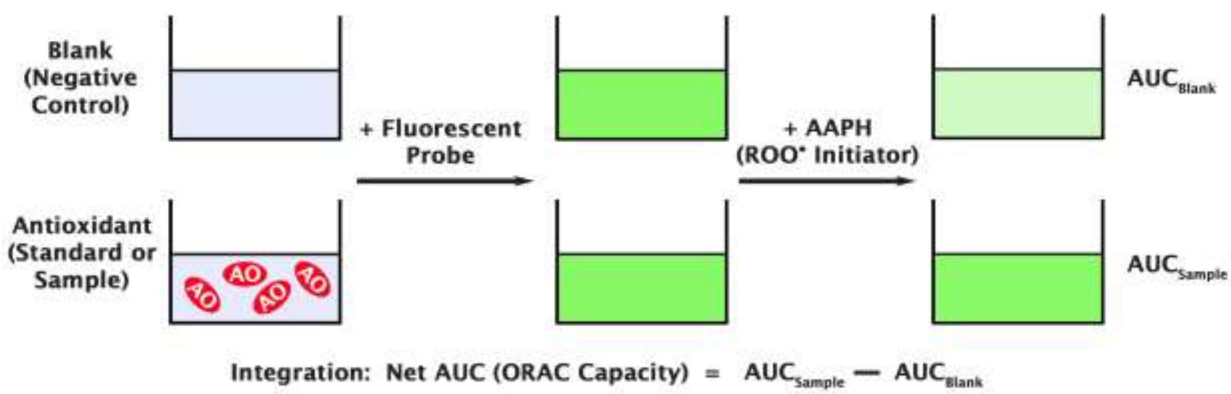
Introduction

Oxidative stress is a physiological condition where there is an imbalance between concentrations of reactive oxygen species (ROS) and antioxidants. However, excessive ROS accumulation will lead to cellular injury, such as damage to DNA, proteins, and lipid membranes. The cellular damage caused by ROS has been implicated in the development of many disease states, such as cancer, diabetes, cardiovascular disease, atherosclerosis, and neurodegenerative diseases. Under normal physiological conditions, cellular ROS generation is counterbalanced by the action of cellular antioxidant enzymes and other redox molecules. Because of their potential harmful effects, excessive ROS must be promptly eliminated from the cells by this variety of antioxidant defense mechanisms. Antioxidants include both hydrophilic and lipophilic molecules for metabolizing ROS.

Although the products of ROS-induced oxidative stress are extensively used to monitor the effects of oxidative stress, it is also important to evaluate the antioxidant capacity of biological fluids, cells, and extracts. The Oxygen Radical Antioxidant Capacity (ORAC) Assay is a classic tool for measuring the antioxidant capacity of biomolecules from a variety of samples. The ORAC Activity 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 of the water soluble vitamin E analog Trolox™ (see Assay Principle below).

Cell Biolabs' OxiSelect™ ORAC Activity Assay is a fast and reliable kit for the direct measurement of ORAC antioxidant capacity from cell lysate, plasma, serum, tissue homogenates, and food extracts. Each kit provides sufficient reagents to perform up to 192 assays, including blanks, antioxidant standards and unknown samples. The assay is designed for use in single plate microplate readers as well as readers with high-throughput capabilities. Please read the complete kit insert prior to performing the assay.

Assay Principle



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Related Products

1. STA-305: OxiSelect™ Nitrotyrosine Protein ELISA Kit
2. STA-310: OxiSelect™ Protein Carbonyl ELISA Kit
3. STA-312: OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit
4. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
5. STA-324: OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP Sites)
6. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
7. STA-337: OxiSelect™ 8-iso-Prostaglandin F2α ELISA Kit (96 Assays)
8. STA-340: OxiSelect™ Superoxide Dismutase Activity Assay
9. STA-341: OxiSelect™ Catalase Activity Assay
10. STA-346: OxiSelect™ HORAC Activity Assay

Kit Components

1. 96-well Microtiter Plate (Part No. 234501): Two 96-well clear bottom black plates.
2. Fluorescein Probe (100X) (Part No. 234502): One 0.5 mL vial.
3. Free Radical Initiator (Part No. 234503): One 0.5 g bottle of powder.
4. Antioxidant Standard (Trolox™) (Part No. 234504): One 100 µL vial of a 5 mM solution.
5. Assay Diluent (4X) (Part No. 234505): One 50 mL bottle.

Materials Not Supplied

1. Sample extracts for testing
2. 1X PBS and Deionized water
3. 50% Acetone
4. 37ºC incubator
5. Bottles, flasks, and conical or microtubes necessary for reagent preparation
6. Reagents and materials necessary for sample extraction and purification
7. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
8. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
9. Multichannel micropipette reservoir
10. Fluorescence microplate reader equipped with a 480 nm excitation filter and 520 nm emission filter

Storage

Upon receipt store the Fluorescent Probe and Antioxidant Standard (Trolox™) frozen at -20ºC. Aliquot as necessary to avoid multiple freeze/thaws. Store all remaining components at 4ºC.

Preparation of Reagents

- 1X Assay Diluent: Dilute the Assay Diluent 1:4 with deionized water. Mix to homogeneity. Use this for all sample and standard dilutions. Store the 1X Assay Diluent at 4ºC.
- 1X Fluorescein Probe: Dilute the Fluorescein Probe 1:100 with 1X Assay Diluent. Mix to homogeneity. Label this as 1X Fluorescein Solution. Use only enough Fluorescein Probe as necessary for immediate applications.

  Note: Do not store diluted Fluorescein Probe solutions.
- Free Radical Initiator Solution: Freshly prepare 80 mg/mL Free Radical Initiator Solution in 1X PBS. For example, 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.

Preparation of Samples

Note: 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 4ºC. Remove the supernatant for measuring the non-protein fraction in the assay.
- Cell Culture: Wash cells 3 times with cold PBS prior to lysis. Lyse cells with sonication or homogenation in cold PBS and centrifuge at 10,000 x g for 10 minutes at 4ºC. 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 or Serum: Collect blood with heparin and centrifuge at 4ºC for 10 minutes. Remove the plasma and aliquot samples for testing. Blood plasma or serum should be diluted 100-fold or more with Assay Diluent prior to performing the assay.
- Tissue Lysate: Sonicate or homogenize tissue sample on cold PBS and centrifuge at 10,000 x g for 10 minutes at 4ºC. 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:
  - Solid or High Protein Samples: Weigh solid sample and then homogenize after adding deionized water (1:2, w/v). Centrifuge the homogenate at 10-12,000 x g for 10 minutes at 4ºC. 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(solid pulp)/v) and mixing at room temperature for 30-60 minutes. Centrifuge the extract/solid at 12,000 x g for 10 minutes at 4ºC. Recover the

acetone extract and dilute with Assay Diluent as necessary prior to running the assay. The total ORAC value is calculated by combining the results from the water-soluble fraction and the acetone extract from the pulp fraction.

- Aqueous Samples: Centrifuge the sample at 5-10,000 x g for 10 minutes at 4ºC 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.

Preparation of Antioxidant Standard Curve

I. Hydrophilic (aqueous) Samples

1. Prepare fresh standards by diluting the 5 mM Antioxidant Standard stock solution to 0.2 mM in Assay Diluent (example: add 10 µL of Antioxidant Standard stock tube to 240 µL of Assay Diluent).
2. Prepare a series of the remaining antioxidant standards according to Table 1 below.

Tubes	0.2 mM Trolox™ Antioxidant Standard (µL)	Assay Diluent (µL)	Resulting Trolox™ Concentration (µM)
1	50	150	50
2	40	160	40
3	30	170	30
4	20	180	20
5	10	190	10
6	5	195	5
7	2.5	197.5	2.5
8	0	200	0

Table 1. Preparation of Standards for use when testing Hydrophilic Samples.

Note: Do not store diluted Antioxidant Standard solutions.

II. Lipophilic Samples

1. Prepare fresh standards by diluting the 5 mM Antioxidant Standard stock solution to 0.2 mM in 50% acetone (example: add 10 µL of Antioxidant Standard stock tube to 240 µL of acetone).
2. Prepare a series of the remaining antioxidant standards according to Table 2 below.

Tubes	0.2 mM Trolox™ Antioxidant Standard (µL)	50% Acetone (µL)	Resulting Trolox™ Concentration (µM)
1	50	150	50
2	40	160	40
3	30	170	30
4	20	180	20
5	10	190	10
6	5	195	5
7	2.5	197.5	2.5
8	0	200	0

Table 2. Preparation of Standards for use when testing Lipophilic Samples.

Note: Do not store diluted Antioxidant Standard solutions.

Assay Protocol

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

1. Add 25 µL of the diluted Antioxidant Standard or samples to the 96-well Microtiter Plate.
2. Add 150 µL of the 1X Fluorescein Solution to each well. Mix thoroughly. Incubate the plate for 30 minutes at 37ºC.
3. Add 25 µL of the Free Radical Initiator Solution into each well using either a multichannel pipette or a plate reader liquid handling system.
4. Mix the reaction mixture thoroughly by pipetting to ensure homogeneity.
5. Immediately begin reading sample and standard wells with a fluorescent microplate reader at 37ºC with an excitation wavelength of 480nm and an emission wavelength of 520nm. 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.

Example of Results

The following figure demonstrates typical OxiSelect™ ORAC Activity Assay results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

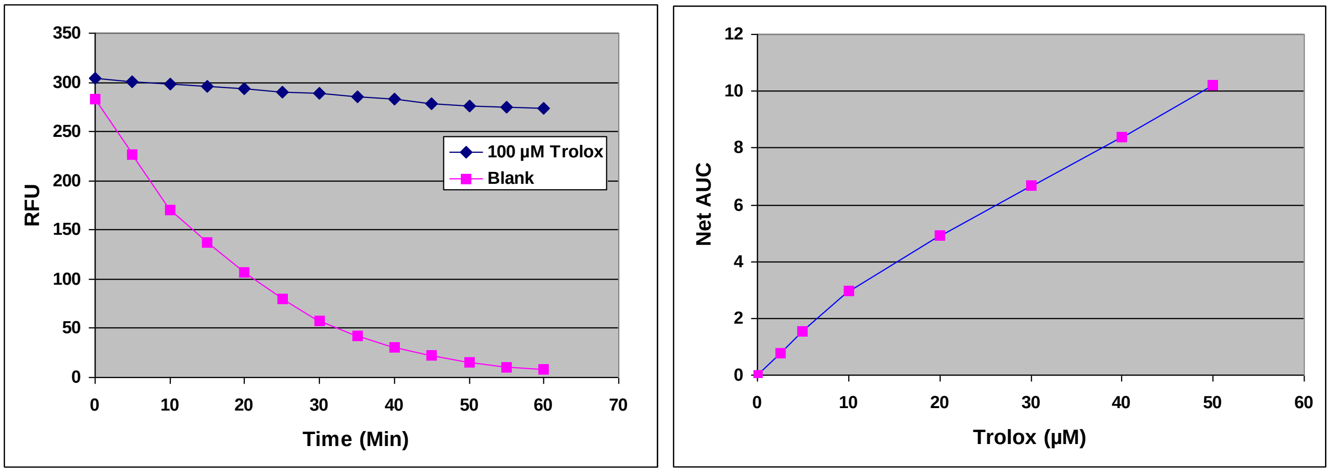


Figure 1: ORAC Activity Assay Standard Curve.

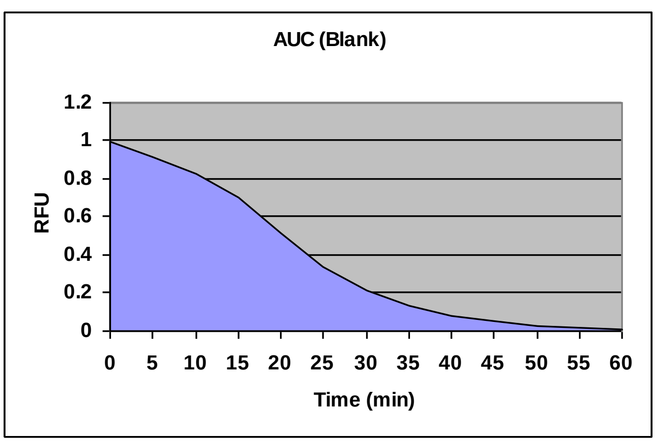
Calculation of Results

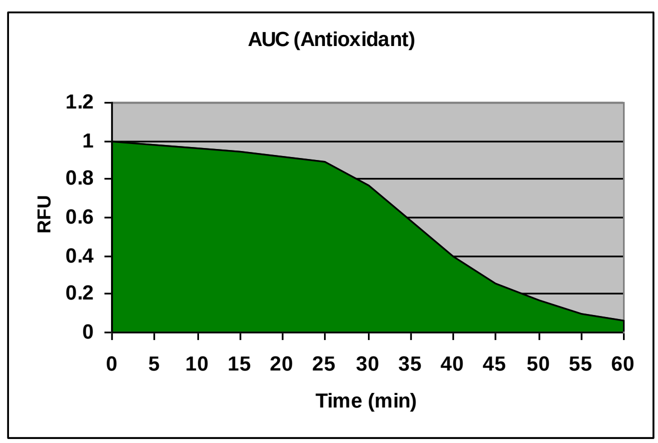
Note: A spreadsheet application or plate reader software can be used to perform the calculations.

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:

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

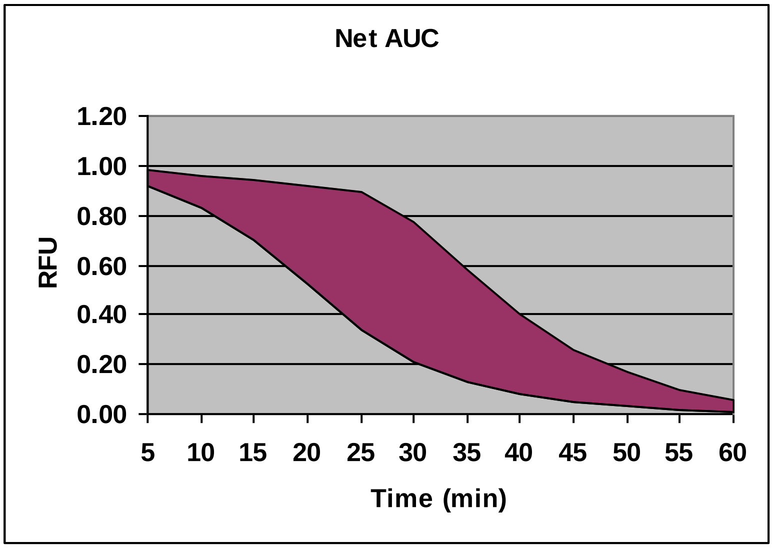
   $\mathbf{RFU_0}$ = relative fluorescence value of time point zero.
   $\mathbf{RFU_x}$ = relative fluorescence value of time points (eg. RFU_5 is relative fluorescence value at minute five)





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 (see Figure 1)
4. Calculate the µMole Trolox™ Equivalents (TE) of unknown sample by comparing the standard curve. Results (ORAC value) may be expressed as TE per L or g of sample.

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 = AUC (Antioxidant) – AUC (blank) = 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

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Warranty

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