

ORAC Assay on the FLUOstar OPTIMA to Determine Antioxidant Capacity

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Application Note 148

Rev. 12/2006



- Antioxidants are able to neutralize Reactive Oxygen Species (ROS)
- Antioxidant capacity successfully determined with the ORAC assay on the FLUOstar OPTIMA
- ORAC assay uses Trolox® as reference substance

Introduction

In all oxygen consuming cells, metabolism and oxidative stress generate several intermediates and byproducts that are collectively known as reactive oxygen species (ROS). ROS are necessary intermediates in the human body, but they are also involved in the aging process and in the development of many degenerative diseases, including cancer, heart disease, Alzheimer's and Parkinson's. ROS are dangerous to cellular structures and functional molecules (i.e. DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. Biological antioxidants are able to dispose of ROS; however, they are not completely effective in eliminating all of the free radicals, oxygen ions and peroxides that can do damage to the body. Furthermore, ROS can be generated from exposure to other external sources such as cigarette smoke, pollutants, chemicals and environmental toxins.

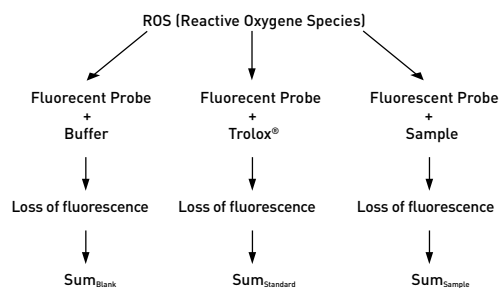
In recent years, clinical trials and epidemiology studies have shown an inverse relationship between the consumption of fruits and vegetables and degenerative diseases¹. This data suggests a correlation between antioxidant laden food and good health; though an inverse relationship with a specific antioxidant (i.e. carotenoid, vitamin C or vitamin E) and a specific disease has not been completely successful. One major drawback to these latter studies has been determining the antioxidant capacity of these foods and their specific molecules, as well as the antioxidant capacity of plasma or other biological samples.

One standardized method for determining the antioxidant capacity of a substance is the ORAC (oxygen radical absorbance capacity) assay². The ORAC assay is based upon the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)]³. In this manner, the ORAC assay uses a biological relevant radical source and it combines both inhibition time and degree of inhibition into one quantity. Recent modifications to this assay include the use of fluorescein as the probe⁴, the adaptation to a high-throughput format, and the ability to measure the lipophilic, hydrophilic, and total⁵ antioxidant capacity of a substance. These modifications, along with no washing steps, have greatly simplified the ORAC assay; thereby making it ideally suited to measure the antioxidant capacity of a substance.

Herein we describe the application of the ORAC-FL assay on a FLUOstar OPTIMA using Trolox® (a water-soluble analogue of vitamin E) as a standard by which all other antioxidant compounds are compared.

Assay Principle

Over time ROS, generated from the thermal decomposition of AAPH, will quench the signal from the fluorescent probe fluorescein. The subsequent addition of an antioxidant produces a more stable fluorescence signal, with signal stability depending on the antioxidant's capacity (Fig. 1). The data points are summarized over the time by the evaluation software. This is then compared to the standard, Trolox®, and is expressed as micromoles of Trolox® equivalents (TE) per gram or per milliliter of sample (µmole of TE/g or µmole of TE/mL).



$$\text{Antioxidant Capacity relating to Trolox}^{\circledR} = \frac{(\text{Sum}_{\text{Sample}} - \text{Sum}_{\text{Blank}})}{(\text{Sum}_{\text{Standard}} - \text{Sum}_{\text{Blank}})}$$

Fig. 1: ORAC Assay Principle

Materials and Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- Costar® 96 well black opaque plate, Corning Costar Corporation, Cambridge, MA, cat. no. 3792
- Fluorescein Sodium, 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox®), L (+)-ascorbic acid, Epicatechin gallate, [2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH)] were obtained from Sigma-Aldrich
- Plate sealer, BMG LABTECH, Aylesbury, UK, Cat. No. 77400-05
- Thermostar, BMG LABTECH, Offenburg, Germany
- FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany



Fig. 2: BMG LABTECH's FLUOstar OPTIMA

Although a FLUOstar OPTIMA was utilized for this application note, BMG LABTECH's POLARstar also have been used for fluorescence measurements.

Test Protocol

Different dilutions of Trolox® (200 µM – 12.5 µM) and sample compounds (ascorbic acid and epicatechin gallate, two known antioxidants) were prepared in phosphate buffer (10 mM, pH 7.4). All solutions were and should be prepared fresh daily.

In every working well the following was pipetted in triplicate:

- Fluorescein, 150 µl of a 10 nM solution
- For standard, 25 µl of Trolox® dilution
- For sample, 25 µl of sample dilution
- For blank, 25 µl of phosphate buffer

The microplates were sealed followed by an incubation for 30 min at 37°C in a ThermoStar microplate incubator without shaking. Alternatively, the FLUOstar OPTIMA itself can perform the incubation step.

After incubation, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 sec to determine the background signal. After 3 cycles, 25 µl (240 mM) of AAPH was injected with the help of onboard injectors. Alternatively AAPH can also be added manually with a multi-channel-pipette. This has to be done as quickly as possible since the ROS-generator displays immediate activity after addition. The test was resumed and fluorescent measurements were taken up to 90 minutes.

Instrument Settings Overview

Mode:	Fluorescence intensity, plate mode
Optic :	Top optic, combination head
Filter:	Exc. 485 nm Em. 520 nm
No. of cycles:	60
Measurement start time:	0.0 sec
No. of flashes:	10
Cycle time:	90 sec
Gain:	Adjusted for each plate

Results

Figure 3 shows Trolox® signal curves (relative fluorescent units versus time) at different concentrations. After 3 cycles AAPH was added, which lead to a loss in fluorescence signal that depended upon the concentration of Trolox®.

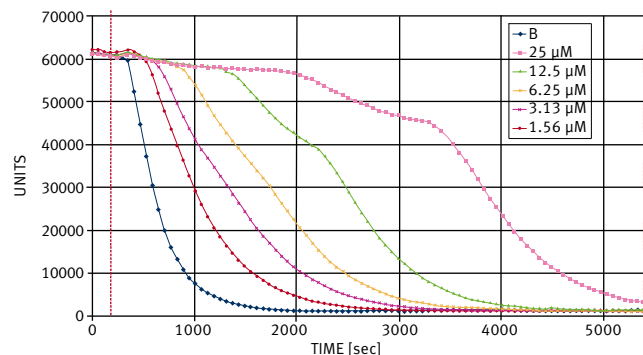


Fig. 3: Signal curves of different concentrations of Trolox® compared to the Blank (blue line).

Since the sample concentrations are known, the software has a feature in layout (found in test protocols setup) that allows the user to simultaneously look at calibration curves from up to 12 compounds. Figure 4 depicts the blank-corrected linear regression curves of Trolox®, ascorbic acid and epicatechin gallate. Graphically one can see that ascorbic acid is a weaker antioxidant than Trolox®, whereas epicatechin gallate is a much stronger one.

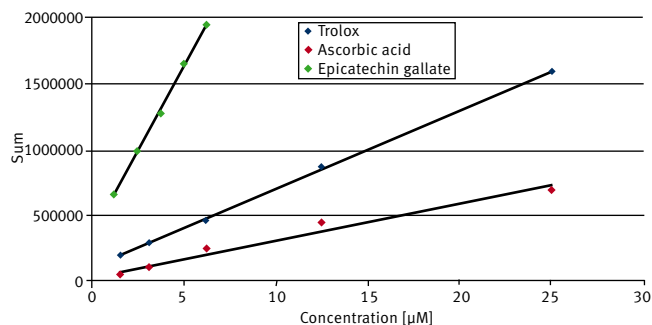


Fig. 4: Blank-corrected linear regression curves of Trolox®, ascorbic acid and epicatechin gallate. The data points were summed over time and were plotted on the y-axis vs. concentration.

To obtain the values for Trolox® equivalents (TE) of antioxidants with known concentration over the desired concentration range one can divide the slopes of the regression curves:

$$\text{TE over considered concentration range} = \frac{\text{slope regression curve (sample)}}{\text{slope regression curve (Trolox®)}}$$

In the case of compounds with unknown concentrations, the software calculates the Trolox® equivalents of a special dilution using the Trolox® calibration curve.

Conclusion

The ORAC assay is a common and popular tool used to determine the antioxidant capacity of any substance. With the help of the FLUOstar OPTIMA and its easy-to-use software, the antioxidant capacity of a substance can be directly estimated by comparison to the standard curve of Trolox®. The progress of each reaction can be followed in real-time using the current state option. Furthermore, the use of onboard injectors allow for consistent and reproducible data.

References

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