BMG LABTECH

ORAC assay to determine antioxidant capacity

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- **Antioxidants are able to neutralize Reactive Oxygen Species (ROS)**
- **ORAC assay uses Trolox® as reference substance**
- **MARS Data analysis software with predefined template for quick ORAC evaluation**

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.

One standardized method for determining the antioxidant capacity of a substance is the ORAC (oxygen radical absorbance capacity) assay. The ORAC assay is based upon the inhibition of the peroxylradical-induced oxidation initiated by thermal decomposition of azocompounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH)]. 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 probe, the adaptation to a high-throughput format, and the ability to measure the lipophilic, hydrophilic, and total 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 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).

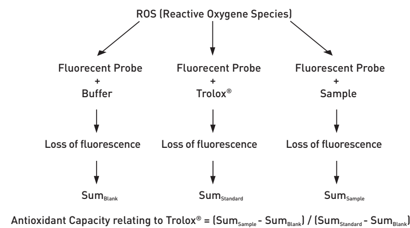

Fig. 1: ORAC Assay Principle.

Materials & Methods

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

- Costar® 96 well black opaque plate, Corning Costar
- Fluorescein Sodium, 6-Hydroxy-2,5,7,8-tetra-methylchroman-2- carboxylic acid (Trolox®), L (+)-ascorbic acid, Epicatechin gallate, [2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH)] were obtained from Sigma-Aldrich

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 incubation for 30 min at 37°C in a Thermostar microplate incubator without shaking. Alternatively, the BMG LABTECH microplate reader itself can perform the incubation step.
After incubation was finished, 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.

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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 120 minutes.

Instrument settings

	FLUOstar®/ POLARstar® Omega	CLARIOstar®	PHERAstar® *FS*
Detection mode	Fluorescence Intensity		
Method	Plate mode kinetic, top optic		
Optic settings	Ex-Filter: Ex485 Em-Filter: Em520	Ex-Filter: Ex485 Em-Filter: Em520 or Monochromator: Ex: 483-14 Em: 530-30	Optic module: FI 485 520
Cycle Time	90 seconds		
Number of cycles	80		

Results & Discussion

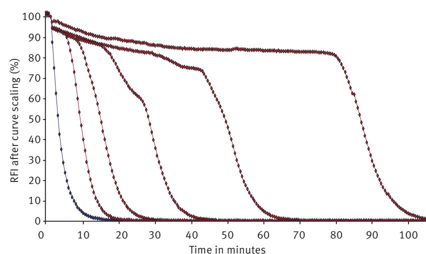

Fig. 2: Signal curves for different Trolox® concentrations (red graphs) and a blank without Trolox (blue graph) recorded on the PHERAstar *FS* in 384-well format. The curves were normalized to 100 %. The 100 % value is the maximum value that is obtained directly after injection of AAPH.

Figure 2 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®.
Since the sample concentrations are known, the software allows the user to simultaneously look at calibration curves. Figure 3 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.

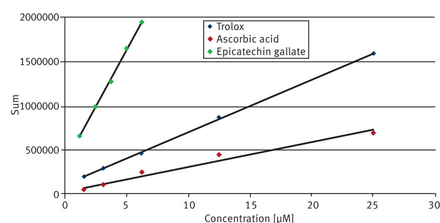

Fig. 3: Blank-corrected linear regression curves of Trolox®, ascorbic acid and epicatechin gallate. The data points were summed over time and were plotted on the y-axis vs. concentration.

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 easy-to-use MARS Data analysis 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 realtime using the current state option. Furthermore, the use of onboard injectors allow for consistent and reproducible data.

PHERAstar® *FSX*

*The PHERAstar *FSX* is the newest PHERAstar reader.

CLARIOstar®

Omega Series