INTRODUCTION

Background

Oxidative stress has been implicated in a number of diseases such as atherosclerosis\(^1\), chronic inflammatory disease\(^2\), chronic renal failure\(^3\) and cancer\(^4\). Oxidative stress is the condition in which an imbalance between oxidant stimuli and physiological antioxidants exist leading to the damage of a cell. The body’s physiological response to oxidative stress is through several antioxidant systems\(^5\) that include enzymes and varying sized molecules (see example below). These antioxidants can be found as water-soluble or lipid soluble molecules and localized transiently throughout tissues, cells and cell types.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes:</td>
<td>superoxide dismutase, catalase, glutathione peroxidase</td>
</tr>
<tr>
<td>Large Molecule:</td>
<td>albumin, ferritin, ceruloplasmin</td>
</tr>
<tr>
<td>Small Molecule:</td>
<td>ascorbic acid, (\alpha)-tocopherol, (\beta)-carotene, uric acid</td>
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</tbody>
</table>

Given the multiplicity of antioxidant pathways, their centrality in the prevention of oxidant stress, and the influences of lifestyle and nutritional supplements on an individual’s antioxidant capacity, it is important to be able to quantitatively measure the total antioxidant capacity or antioxidant power within biological specimens\(^6-11\).

PRINCIPLE OF ASSAY

The combined action of the antioxidants provided by the sample or standard acts to reduce Cu\(^{2+}\) to Cu\(^+\). This reduced form of copper will selectively form a 2:1 complex with the chromogenic reagent. This complex is stable and has an absorption maximum at ~ 450 nm\(^12-16\). A known concentration of Trolox is used to create a reference curve to compare those readings obtained by the samples. Data can be expressed as mM Trolox equivalents or in \(\mu\)M copper reducing equivalents.

Results of a study performed to evaluate the correlation between the concentration of principal antioxidants in human serum and the value obtained for Total Antioxidant Power using this method are presented in Figure 1. (Multivariate analysis of the obtained results yields a very high significance.)

Results obtained for the Total Antioxidant Power for a series of serum samples were also compared to the resistance to oxidation of the serum lipids in these samples. The results of this study also show that these
two parameters are highly correlated. The higher the Total Antioxidant Power, the more protected are the serum lipids to oxidation.

Figure 1. Correlation between antioxidant concentration and reduction from Cu$^{2+}$ to Cu$^+$ using this method.

![Graph showing correlation between antioxidant concentration and reduction from Cu$^{2+}$ to Cu$^+$](image)

MATERIALS PROVIDED

1. DILUTION BUFFER: 60 mL of dilution buffer for the dilution of both sample and standard.  
2. COPPER SOLUTION: 5 mL of Cu$^{2+}$ Solution.  
3. STOP SOLUTION: 5 mL of stop solution.  
4. TROLOX STANDARD: 1 lyophilized vial of 2 mM Trolox standard.  
5. MICROPLATE: One optically clear 96 well microplate.

MATERIALS NEEDED BUT NOT PROVIDED

1. Adjustable pipettes (10-1,000 μL) and disposable tips.  
2. Double deionized water (DD water).  
4. Vortex mixer.
STORAGE CONDITIONS

Store the components of this kit at the temperatures specified on the labels. Aliquot the unused portion of the reconstituted TROLOX STANDARD and store at -80°C until kit expiration.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents from individual vials.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents is not recommended as it may be detrimental to the assay.
3. Gloves and lab coat should be worn at all times while performing this assay. Contents may be harmful if swallowed, inhaled or absorbed through the skin.
4. Chelating agents such as EDTA and sodium citrate should be avoided in the use of this kit.

SAMPLE PREPARATION

All samples should be stored at -70°C at all times prior to assay. Apply samples to the assay immediately upon thawing. Samples with a Trolox equivalent concentration greater than 2.0 mM should be diluted with pH 7.0 PBS prior to assay.

Sample preparation is subject to the discretion and approval of the principal investigator. Deviations may occur to optimize or better complement experimental design.

NOTE: Chelators such as EDTA may be detrimental to the function of the assay and should be avoided in the use of this kit.

Tissue Lysate: Homogenize tissue sample on ice with ice cold pH 7.0 PBS then centrifuge at 3000 x g for 12 minutes at 4°C. Aliquot the supernatant for -70°C storage, protein determination, and Total Antioxidant Assay.

Cell Culture: Wash cells 2-3 times with ice cold pH 7.0 PBS prior to lysis. Lyse cells by homogenization or sonication with ice cold pH 7.0 PBS then centrifuge at 3000 x g for 12 minutes at 4°C. Aliquot the supernatant for -70°C storage, protein determination, and Total Antioxidant Assay.

Urine: Collected samples may be assayed directly or diluted with pH 7.0 PBS where appropriate.

Plasma: Collect blood with sodium citrate and centrifuge at 3000 x g 4°C for 12 minutes. Remove plasma and aliquot for -70°C storage, protein determination, and Total Antioxidant Assay. Sample results can alternatively be expressed in mL of plasma.

Food: This can vary considerably from one food to the next and requires foresight and discretion from the principal investigator. In many cases liquid food samples such as juice and tea can be assayed directly without any processing. Liquid samples that contain high amounts of protein and/or fiber or solid foods should be processed as indicated below.
Solid Food: Homogenize food sample on ice in ice cold pH 7.0 PBS then centrifuge at 3000 x g for 12 minutes at 4°C. Aliquot the supernatant for -70°C storage, protein determination, and Total Antioxidant Assay.

Liquid Food: Centrifuge at 3000 x g for 12 minutes at 4°C. Aliquot the supernatant for -70°C storage, protein determination, and Total Antioxidant Assay.

STANDARD PREPARATION

PLEASE NOTE: Trolox standard vial is under vacuum.

TO RECONSTITUTE THE TROLOX STANDARD:

Preferred Method – Add 1.5 mL of DD water directly to the Trolox standard vial with a needle and syringe by puncturing the rubber stopper with the needle. Vortex the vial for 30-60 seconds until the standard is completely dissolved. The concentration is now 2.0 mM. Slowly remove the rubber stopper allowing the vacuum seal to break and proceed to Table 1 for the standard curve dilutions.

Alternative Method – WARNING!!! The solid Trolox can become airborne due to the opening process or due to the turbulence created during pipetting and reconstitution. Loss of material will greatly affect the accuracy of your assay – this method is performed at the users risk and discretion.

Slowly lift a corner of the rubber stopper until the vacuum seal is broken. Now remove the stopper and slowly add 1.5 mL of DI water to the internal side of the vial. Recap the vial with the same rubber stopper and parafilm or tape the stopper to the vial then vortex for 30-60 seconds until the standard is completely dissolved. The concentration is now 2.0 mM remove the stopper and proceed to the standard curve scheme.

The reconstituted standard can be aliquotted and stored at –70°C for up to one year.

Table 1. Standard Curve Preparation

<table>
<thead>
<tr>
<th>Standard</th>
<th>Trolox Conc. (mM)</th>
<th>Vol. of Deionized Water (uL)</th>
<th>Transfer Volume (uL)</th>
<th>Transfer Source</th>
<th>Final Volume (uL)</th>
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<tr>
<td>S5</td>
<td>2</td>
<td>-</td>
<td>2000</td>
<td>2 mM Stock</td>
<td>1500</td>
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<tr>
<td>S4</td>
<td>1</td>
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<td>500</td>
<td>S5</td>
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<td>1000</td>
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<tr>
<td>S0</td>
<td>0</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>500</td>
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ASSAY PROCEDURE

Allow dilution buffer, copper solution and stop solution to equilibrate to room temperature for 30 minutes prior to running the assay. Dilute both sample and standards 1:40 in the provided dilution buffer (e.g. 15 μL serum + 585 μL buffer). See Scheme I for suggested plate layout.

1. Place 200 μL of diluted samples or standards in each well. Reagent blanks should be dilution buffer provided in the absence of standard or sample.
2. Read the plate at 450 nm for a reference measurement.
3. Add 50 μL of Cu solution to each well and incubate 3 minutes at room temperature.
4. Add 50 μL of stop solution.
5. Read the plate a second time at 450 nm.

Scheme I.

<table>
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<tr>
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<td>U3</td>
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</table>

INTERPRETATION OF RESULTS

1. Calculate the net absorbance by subtracting the OD reading from Step 3 from the reading in Step 5.
2. Plot the net absorbance vs. the Trolox concentration. See representative standard curve depicted in Figure 2.

Samples can be expressed in terms of μM Trolox equivalents by solving for “x” from your generated y-intercept equation. See example below. To express values in μM copper reducing equivalents, multiply the “x” value coefficient by 2189 μM.
y = mx + b where:
  y = The ordinate or y-axis value (corresponds to the OD reading)
  x = The abscissa or x-axis value (corresponds to the Trolox concentration)
  m = Slope
  b = Intercept

To express values in \( \mu M \) copper reducing equivalents (CRE’s), multiply the “x” value coefficient by 2189 \( \mu M \).

To express values in \( \mu M \) Uric Acid equivalents, divide the \( \mu M \) Trolox equivalents by 1.33

Figure 2. Typical Standard Curve

REFERENCES

DISCLAIMER

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