コスモ・バイオ株式会社

Applied Bioanalytical Labs

TAC-Peroxyl

A Chemiluminescence Test Kit for Total Antioxidant Capacity against Peroxyl Radicals

(For 30 test tube assays or 100 microplate assays)

Store at 2 – 8°C

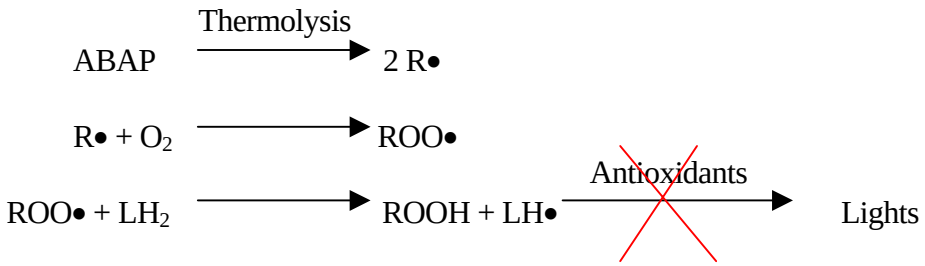
For *In-Vitro* Research Use Only

Introduction

Reactive oxygen free radicals (ROS) have been implicated in more than 100 human diseases and in aging process.[1] Tissue damage caused by free radicals is also well documented in trauma, toxin shocks, ischemia/reperfusion. ROS are generated endogenously by aerobic respiration, inflammation and lipid peroxidation, to name a few. Exogenously generated ROS pose un-precedent challenge to human kind because of deteriorating environment, tobacco smoking, ionization radiation, UV-light exposure, organic solvents, anesthetics, pesticides and medications. Organisms have developed a powerful antioxidant defense system to minimize or prevent deleterious effects from ROS exposure.[1] Enzymes such as superoxide dismutase, glutathione peroxidase and catalase aid in the decomposition of harmful radical species. Small free radical-scavenging molecules such as ascorbic acid, glutathione, uric acid, vitamin-E and CoQ-10 act as free radical scavengers. Macromolecules work to chelate metals and adsorb free radicals. The overall antioxidant status is also related to other factors such as disease, life-style (exercise and diet) and an organism's stress load in general.

Numerous methods have been described in literature to evaluate total antioxidant capacity (TAC) of samples.[2] These methods can be categorized into scavenging assays against 1) superoxide anion radical, 2) hydrogen peroxide, 3) hypochlorous acid, 4) hydroxyl radical, 5) peroxyl radicals, 6) peroxylnitrite. There are also methods using less biologically relevant systems such as those measuring capacity to reduce ferric ion and cuperic ion, and those measuring scavenging ability toward 2,2-diphenyl-1picryhydrazyl (DPPH) radical and towards N,N-dimethyl-p-phenyleneamine (DMPD) radical. These methods tend to give varied results because one particular antioxidant compound has differed ability to scavenge a given free radical, and in a complex biological samples yields different contributions to the total antioxidant capacity in the different assay platforms. Therefore, it is important to describe TAC data in terms of a specific system used.

Our assay is based on inhibition of luminescence caused by peroxyl radicals that is involved in lipid peroxidation *in vivo*.[2-4] This assay is the most popular method for the analysis of a wide range of biological samples such as serum (plasma), CSF, semen plasma, tissue homogenates and urine. And it can also been used in the analysis of tea, wine, fruits and botanical extracts. The platform of this kit[2,3] is an artificial system where biologically relevant peroxyl free radicals are generated by thermal decomposition of 2,2'-azobis(2-amidinopropane) (ABAP). The ABAP decomposition products are a pair of C-centered free radicals R• and a nitrogen molecule. The R• free radicals further react with oxygen molecules to form peroxyl radicals ROO•, which are similar to those found *in vivo* during lipid peroxidation. These peroxyl radicals react with an indicator molecule, luminol (LH_2), to generate a luminol radical (LH•) that results in emission of blue lights centered at ~425 nm. When antioxidants (non-enzymatic) are present, such a light production is inhibited until the antioxidants are exhausted. The inhibition mechanism may be complex, however, antioxidants seem to trap the luminol derived radical at the low concentration range due to efficiency of peroxyl-luminol reaction. The time of inhibition or the induction time to light production is proportional to the total concentration of antioxidants. The antioxidant concentration is determined by comparing induction time to that of a water-soluble Vitamin E (tocopherol) analog, Trolox. The principle of this assay is shown in the following scheme:



Reagents

Materials Provided (for 30 test tube test or 100 microplate test):

Assay Buffer	30 mL
Luminol Solution	1 mL
ABAP	2 vials
Trolox Standard	2 vials
Sample Dilution Buffer	30 mL

Items Required but Not Provided:

Luminometer (single tube or plate reader, temperature control module recommended but not absolutely required) or scintillation counter
Disposable glass or transparent plastic tubes or scintillation vials or white microplate
Microcentrifuge tubes
Plastic or glass bottles
Pipetters, adjustable 0.0 – 1.0 mL
Disposable pippette tips

Storage and Handling

The kit should be stored as a whole in the original box at 2 – 8^{o}C. Return to storage immediately after use. Dispose solution after kit's expiration date.

Preparation of solutions

The assay is very sensitive to temperature. The experiment can only be carried out in an environment where temperature is constant when a single tube luminometer is used so that the results of standards and samples can be compared. This requirement for luminsecence plate reader is less restrictive since assays are carried out in parallel. When there is significant fluctuation of temperature in the experiment or when quick/less-than-perfect result is needed, an alternative sample preparation and assay procedure can be used (see Section Alternative Procedure and Calculation). Let kit warm up to room temperature completely (~1 hour). ABAP and Trolox need to be reconstituted with solvents before the experiment. Reconstituted ABAP and Trolox are not suitable for long-term storage, therefore two sets of vials of ABAP and Trolox are provided for assays performed at separate times.

ABAP:

Add 1.0 mL Assay Buffer to one ABAP vial, mix to dissolve completely. Let it stand at room temperature for 45 minutes before use.

Trolox Standards *(Note: It is normal for standard vials to appear empty)***:**
Add 1000.0 µL of Sample Dilution Buffer to one Trolox vial, mix to dissolve completely. The resultant solution contains 30 µM of Trolox. Dilute this standard in additional three microcentrifuge tubes by mixing following:

Standard No.	Sample Dilution Buffer (µL)	30 µM Standard (µL)	Standard Conc. (µM)
1	375	75	5.0
2	300	150	10.0
3	150	300	20.0
4	0	475	30.0

Samples:
Samples typically need to be diluted with the Sample Dilution Buffer, so that their total antioxidant concentration is in 5 – 30 µM range in the standard set. Following are the general guidelines of dilution required (samples from patients or abnormal conditions may require a different dilution factor):
Human Plasma: 1/20x
Human Semen Plasma: 1/40x
Tissues (10% homogenates): 1/20x (liver, kidney), 1/10x (brain) and 1/2x (heart)
Red Wine: 1/2000x
Brewed Tea: 1/2000x (1 tea bag/100 mL boiling water) or 1/200x (1.5 gram tea leaves/250 mL boiling water).

If the concentration is determined to be out of standard range, adjust the dilution factor and make a new dilution. It is also possible by adding a smaller volume of the diluted sample and making up the total sample volume required with the Sample Dilution Buffer directly in the assay mixture specified in the Procedures. For example, instead of adding 40.0 µL of a secondary 1/10x dilution of a diluted sample, one may add 4.0 µL of the diluted sample and 36.0 µL of Sample Dilution Buffer). In microplate format, several different dilutions of a single sample may be prepared so that at least one dilution will be in the 5 – 30 µM range of the standard set within a single run.

Procedures

For single tube luminometers or scintillation counters:
1. Set measurement temperature slightly (~0.2^{o}C) above room temperature if the luminometer has a temperature controller. Otherwise, carry out the experiment at room temperature.
2. To a clean tube/vial used for luminescence measurement, add 890.0 µL of Assay Buffer. Add 20.0 µL of Luminol Solution, mix. Add 40.0 µL of standard or sample, mix. Incubate for several minutes if the temperature controller is used.
3. Add 50.0 µL of ABAP to start generating free radicals (use automatic injector if available). Mix quickly and start recording luminescence reading immediately every 5 seconds or at a smaller time interval. Take notes of delay time (if any) of the first data point from ABAP addition. Stop recording when the luminescence reaches a maximal plateau or at 30 minutes.

For luminescence plate readers:
1. Set measurement temperature slightly (~0.2^{o}C) above room temperature if the luminescence plate reader has a temperature controller. Otherwise, carry out experiment at room temperature.
2. Use a clean ***white*** 96-well plate. To each well used for test, add 260.0 μL of Assay Buffer. Add 7.0 μL of Luminol Solution to each well. Add 13.0 μL of standard or sample, repeat withdrawing and pipetting a few times to mix. Make notes of standard/sample layout. Incubate for several minutes if temperature controller is used.
3. If the luminometer is equipped with an automatic injector, load ABAP solution (pool two vials of ABAP for whole plate assay) and then inject 17.0 μL of ABAP solution. Otherwise, use a multi-channel pipettor to add ABAP solution to same row or same column simultaneously (use only limited (<4) rows or columns, the delay time is too high for the whole plate manual assay). Take notes of time delay to the next row or column for correcting time difference in measuring induction times. Start recording luminescence reading immediately every 5 seconds or at a smaller time interval. Stop recording when the luminescence reaches the last maximal plateau or at 30 minutes.

Typical Results and Calculations:

A typical standard curve of several Trolox standards is shown below in Figure 1. Without Trolox (antioxidant), the luminescence signal is very strong and decays slightly with time. Trolox almost completely extinguishes this luminescence. When the antioxidant is exhausted (induction time), luminescence shows rapid increase to reach a plateau and then will decay slowly. We use the x-axis interception of the fast-changing part of time course curve to measure the induction time. Using different known concentrations, a standard curve can be obtained and the resultant linear regression equation can be used to calculate antioxidant concentrations (in Trolox equivilent) in samples to be measured. The luminescence intensity sometime is not completely extinguished when some antioxidant samples are used. Then we use the remaining background line instead of x-axis to find the induction time (intercept time point of the fast-changing part of time course curve, see Figure 2).

Some prior publications used time point where a 10% of plateau intensity is reached as the induction time. The induction time measured in this manner is very close to our measurement. Users have some freedom to choose a method to define the induction time, however, consistence is required for same type of samples to provide a basis of comparison.

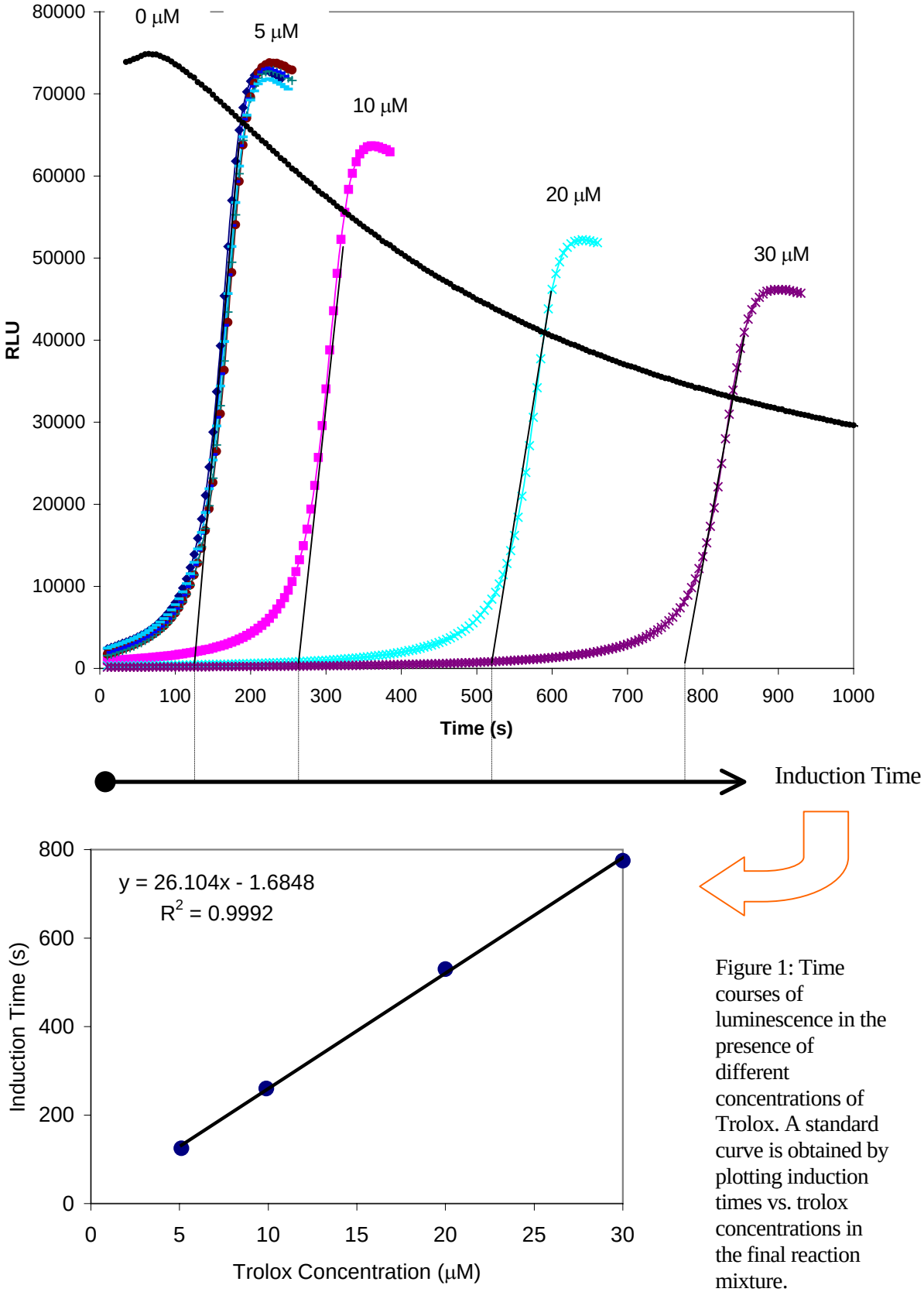


Figure 1: Time courses of luminescence in the presence of different concentrations of Trolox. A standard curve is obtained by plotting induction times vs. trolox concentrations in the final reaction mixture.

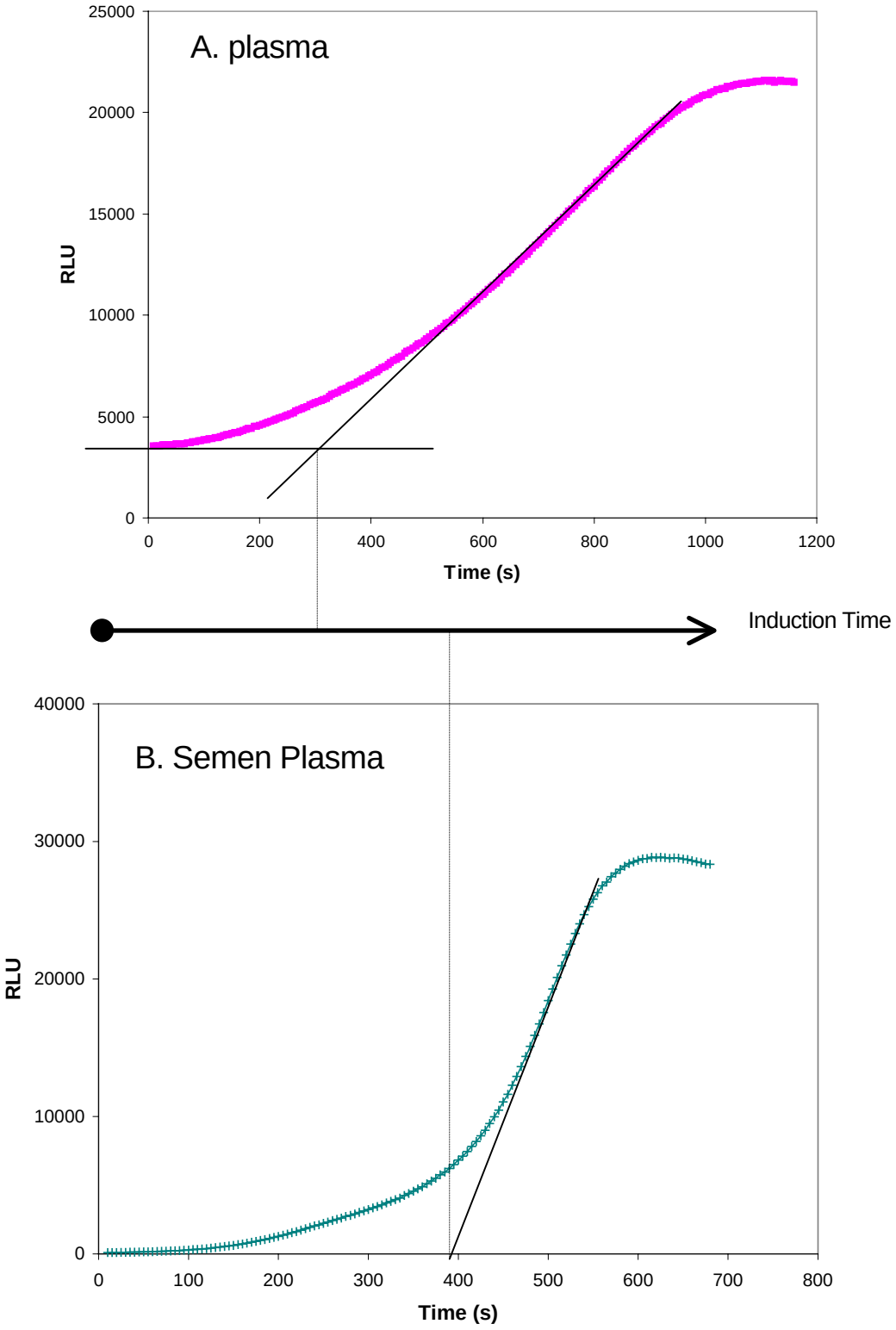


Figure 2. Induction times of a human plasma sample (A) and a human semen plasma sample (B).

Calculations:

1. Measure induction times of standards as illustrated in Figure 1. Also measure and record the induction time of each sample (Figure 2). Make certain the delay times are added back to induction times.
2. Plot induction time (y-axis) vs concentration of trolox (x-axis). Perform a linear regression to get a standard curve:

$$y = ax + b$$

3. The antioxidant concentration (trolox equivalent) in the diluted samples used for the assay is:

$$C_i = (T_i - b)/a$$

where T_i is the induction time of sample i.

4. The antioxidant concentration (trolox equivalent) in the original sample is

$$C^O_i = C_i * (\text{Dilution Factor}_i)$$

Alternative Procedure and Calculation:

With a single tube luminometer without temperature control module, when temperature fluctuates in the laboratory, experiments can be carried out with a standard (e.g., 15 μM Trolox) and a sample (diluted) back-to-back. This procedure is also suitable when a quick and less-than-perfect result is needed. The volumes of reagents used are the same as those described in the Procedures for single tube luminometers and for luminescenece plate readers. The total antioxidant concentration of the diluted sample is calculated as following:

$$C_i = (T_i/T_s) * C_s$$

where T_s and C_s are the induction time and concentration of the standard.

And the concentration of the total antioxidant in the original sample is:

$$C^O_i = C_i * (\text{Dilution Factor}_i)$$

Specifications (Performance Characteristics for Single-tube Luminometer Assays with the Standard Procedure):

LLD: The lower limit of detection is 0.5 μM Trolox.
Linearity: Excellent linearity is maintained up to 30.0 μM of Trolox. The up limit of the linearity is not determined due to long induction time.
Assay Precision: The intra-assay coefficient of variation (CV) is 3.2% for the 5.0 μM Trolox standard.
Recoveries: The recoveries for spiked Trolox in human plasma and human semen plasma are 85% and 104%, respectively.

References

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2. Evelson P. et al., "Evaluation of Total Reactive Antioxidant Potential (TRAP) of Tissue Homogenates and Their Cytosols", *Arch. Biochem. Biophys. 388(2)*, 261-266 (2001).
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4. Alho H. & Leinonen J., "Total Antioxidant Activity Measured by Chemiluminescence Methods", *Methods in Enzymology 299*, 3-15 (1999).

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