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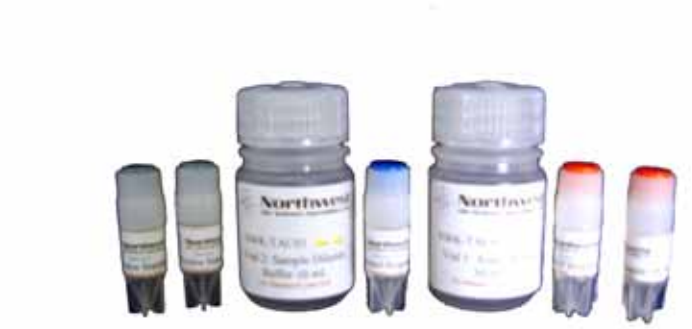
NWLSS™
Total Antioxidant Capacity
TAC-Peroxyl Assay

Product NWK-TAC01
For Research Use Only



Simple assay kit for quantitative measurement of a sample's "Total Antioxidant Capacity" against the biologically relevant peroxyl (ROO•) radical.

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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, toxic shock and ischemia/ reperfusion injury. ROS are generated endogenously by various pathways including aerobic respiration, inflammation and lipid peroxidation. Exogenously generated ROS pose an unprecedented challenge to organisms because of environmental deterioration, tobacco smoking, ionization radiation, UV-light exposure, organic solvents, anesthetics, pesticides and medications. Organisms have developed powerful antioxidant defense systems to minimize or prevent possible deleterious effects of ROS exposure.[1] Enzymatic systems such as superoxide dismutase, glutathione peroxidase and catalase aid in the decomposition of harmful radical species. Small 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 helping to further reduce possibly damaging effects. The overall antioxidant status is also related to other factors such as disease, life-style and an organism's stress load in general. Numerous methods have been described to evaluate the total antioxidant capacity (TAC) of samples.[2] These methods include scavenging assays that challenge a sample with superoxide anion radical, hydrogen peroxide, hypochlorous acid, hydroxyl radical, peroxyl radicals or peroxylnitrite. There are also methods using less biologically relevant systems such as those measuring a sample's capacity to reduce ferric ion and cuperic ion as well as those measuring a sample's scavenging ability toward 2,2-diphenyl-1picryhydrazyl (DPPH) radical and towards N,N-dimethyl-*p*-phenyleneamine (DMPD) radical. Since individual antioxidants differ in scavenging ability toward specif ROS species, it is important to note that TAC data generated using different assay platforms can vary to a significant degree. Because of this fact, it is best to describe TAC data in terms of a specific ROS challenge species.

Intended Use:

The NWLSS™ TAC-Peroxyl assay is designed to evaluate total non-enzymatic antioxidant capacity toward peroxyl radical challenge in biological samples. Applicable sample types include blood plasma or serum, CSF, tissue biopsy, semen plasma, cell lysates, wine, fruit juices, brewed teas, and other botanical extracts.

Test Principle:

Among peroxyl radical based antioxidant assays, total radical-trapping antioxidant parameter (TRAP) methods are frequently cited. These methods include the often cited fluorescent based ORAC assay which measures the ability of a sample to preserve fluorescence during ROS challenge. Unlike the ORAC assay the NWLSS™ TAC-Peroxyl method utilizes free radical mediated induction of luminescence as the standard of measure.[2-4]

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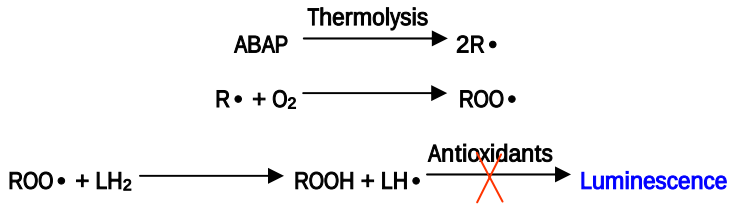
Test Principle (continued):

The platform of the NWLSS™ TAC01 kit is an artificial system where biologically relevant peroxyl free radicals are generated by thermal decomposition of 2,2'-azobis(2-amidinopropane) (ABAP)[2,3]. 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 biologically relevant 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 light centered at ~425 nm. When nonenzymatic antioxidants are present, luminescence is inhibited until the antioxidants are exhausted. 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:



General Specifications:

Format: 1 X 96 wells

Number of tests: 40 duplicate sample tests
24 triplicate sample tests

Specificity: Antioxidant capacity toward peroxyl radical challenge

Sensitivity: LLD = 0.5 μM Trolox equivalents

1. Halliwell B. & Gutteridge J. M. C., "Free Radicals in Biology & Medicine", 3rd Ed., Oxford University Press (New York, 1999).

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).

3. Lissi E. et al., "Evaluation of Total Antioxidant Potential (TRAP) and Total Antioxidant Reactivity from Luminol-Enhanced Chemiluminescence Measurements"", *Free Radic. Biol. Med. 18(2),* 153-158 (1995).

4. Alho H. & Leinonen J., "Total Antioxidant Activity Measured by Chemiluminescence Methods", *Methods in Enzymology 299,* 3-15 (1999).

Statement of Limited Warranty:

Northwest Life Science Specialties, LLC (NWLSS) makes no guarantee of any kind, expressed or implied, that extends beyond the description of the material in this kit, except that they will meet our specifications at the time of delivery. Customer's remedy and NWLSS' sole liability is limited to, at NWLSS' option, refund of the purchase price, or the replacement of material not meeting our specification. By acceptance of our product, customer assumes all liability and will indemnify and hold NWLSS harmless for the consequence of this product's use or misuse by the customer, its employees, or others. Refund or replacement is conditioned of customer notifying NWLSS within twenty-one (21) days of the receipt of product. Failure to give notice within 21 days shall constitute a waiver by the customer of all claims hereunder with respect to said product.

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Data Analysis (cont.):
For some sample types, the luminescence intensity may not be completely extinguished. In these cases we recommend using the remaining background as a baseline (instead of the x-axis); see Figure 2.

Some prior publications have used a 10% of plateau intensity time point as the induction time. The induction time measured in this manner is very close to our maximum rate of change based measurement. End-users have some freedom to choose which method they prefer to use in defining induction time; however, consistency is required for accurate comparison of sample data obtained.

Performance Details:

Stability	All unopened reagents are stable until the expiration date stated on the package label when stored at 2-8°C.
Sensitivity:	The lower limit of detection is 0.5 µM Trolox.
Dynamic Range:	0.5—30 µM Trolox Equivalents in the assay.
Linearity:	Excellent linearity is maintained up to 30.0 µM of Trolox. The upper limit of the linearity was not determined due to long induction times.
Assay Precision:	The intra-assay coefficient of variation (CV) is 3.2% for the 5.0 µM Trolox Standard.
Recovery:	The recoveries for spiked Trolox in human plasma and human semen plasma are 85% and 104%, respectively.

Things to Note:
Temperature fluctuations are the single most likely element to contribute to poor data generation. End users are cautioned to take note of temperature variations when comparing data from separate experiments.

Kit Contents:

Assay Buffer:	1 X 30 mL bottle
Sample Dilution Buffer:	1 X 30 mL bottle
Luminol Reagent:	1 X 1 mL vial
ABAP Reagent:	2 vials
Trolox Standard:	2 vials

Required Materials Not Provided:
White microplate, disposable glass or transparent plastic tubes, else scintillation vials.
Microcentrifuge tubes.
Plastic or glass bottles.
Adjustable pipetters (0.0 – 1.0 mL).
Disposable pippette tips.

Required Instrumentation:
Luminometer (single tube or plate reader, temperature control module recommended but not absolutely required) or scintillation counter.

Warnings, Limitations, Precautions:
The assay is very sensitive to temperature. When a single tube luminometer is used, the experiment should only be carried out in an environment where the temperature is constant assuring that standard and sample results can be accurately compared.

Requirements for a luminsecence plate reader are less restrictive since assays are carried out in parallel. However, when a multi-channel pipettor is used for ABAP reagent addition, the number of wells should be limited to no more than 3 rows because the delay time is too high to perform an entire 96 well plate manual assay. We recommend noting the time delay in next well reagent addition in order to correct for differences in elapsed time for recorded induction times.

When significant temperature fluctuations are expected or when quick, approximate estimates are needed, an alternative sample preparation and assay procedure can be used (see 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.

Storage Instructions:
Upon receipt, store the reagents at 2-8ºC. Do not use components beyond the expiration date printed on the label.

All reagents should be brought to room temperature (20-25ºC) prior to use.

Assay/Instrument Preparation:
Luminescence Plate Reader Setup
Set measurement temperature slightly (~0.2ºC) above room temperature if the luminescence plate reader has a temperature controller. Otherwise, carry out experiment at room temperature.
Single Tube Reader or Scintillation Counter Setup
If the luminescence plate reader has a temperature controller set the measurement temperature slightly above (~0.2ºC) room temperature . Otherwise, carry out experiment at room temperature.

Reagent Preparation:
ABAP Reagent
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 vials to appear empty)
Add 1.0 mL of Sample Dilution Buffer to one Trolox vial; vortex to mix and dissolve completely. The resultant concentration is 30 µM Trolox.. Dilute this standard in three additional microcentrifuge tubes by mixing as follows:

Standard #	Sample Dilution Buffer (µL)	30 µM Standard (µL)	Standard Concentration (µM)
1	375	75	5
2	300	150	10
3	150	300	20
4	0	475	30

Sample Handling/Preparation:
The multi-disciplinary interest in measuring Antioxidant Status has resulted in a myriad of sample types and experimental conditions and is beyond the scope of this product insert to describe sample processing in detail for each case. However, general guidelines are provided below for representative sample types.

Samples typically need to be diluted with Sample Dilution Buffer prior to assay such that their measured antioxidant concentration is within the range of the standards (5–30 µM Trolox equivalents). For convenience we have provided suggested guidelines for starting dilutions. However, experimental samples may require additional or less dilution. It is up to the end user to determine the optimal dilution for their specific model system.

Data Analysis (cont.):

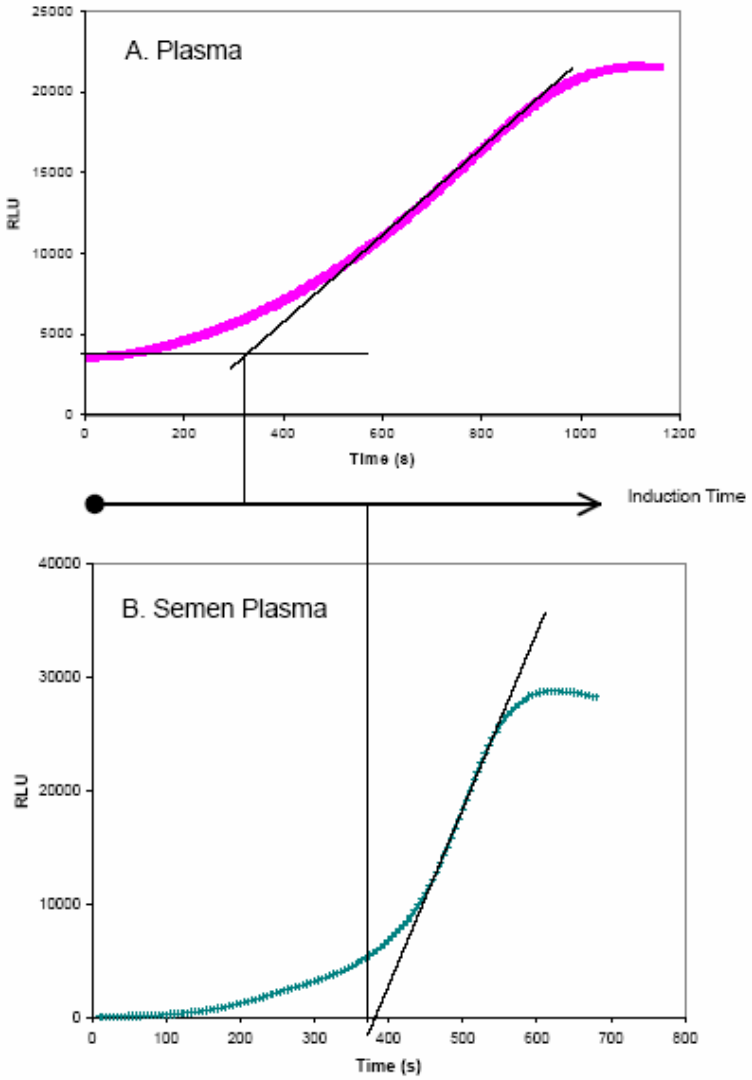

Figure 2. Induction times of a human plasma sample (A) and a human semen plasma sample (B).

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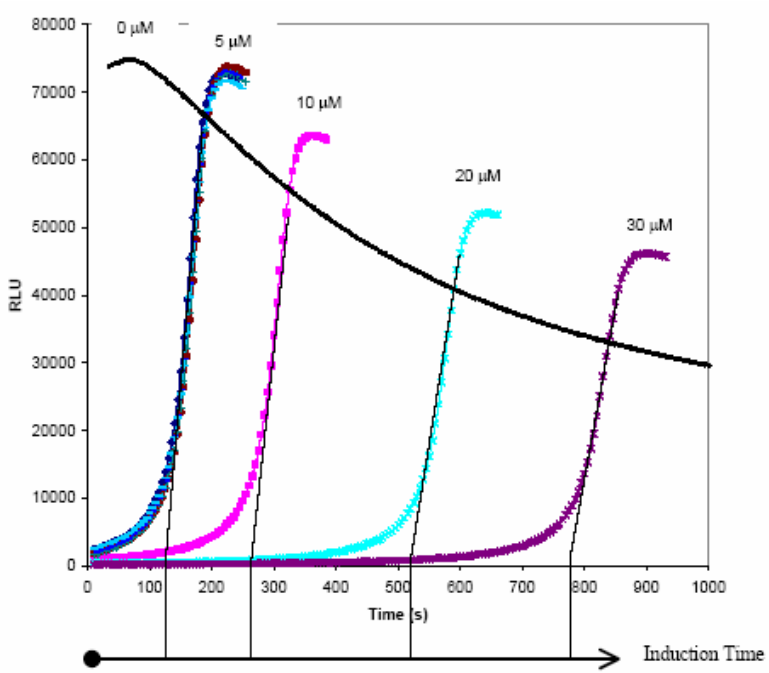
Data Analysis (cont.):



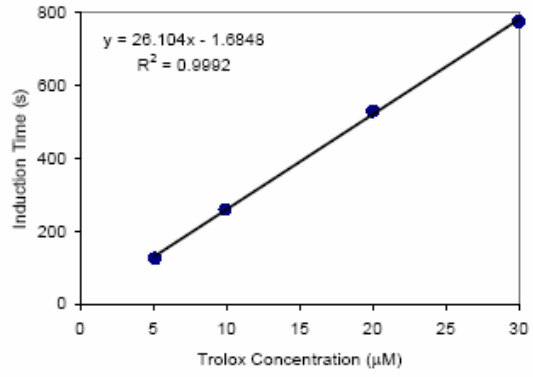


Figure 1: Time courses of luminescence in the presence of different concentrations of Trolox. A standard curve is obtained by plotting induction times vs. trolox concentrations in the final reaction mixture.

Sample Handling/Preparation (cont):

Suggested Dilutions:

Human Plasma:	1/20x
Tissues (10% homogenates):	
Liver or Kidney:	1/20x
Brain:	1/10x
Heart Muscle:	1/2x
Human Semen Plasma:	1/40x
Red Wine:	1/2000x
Brewed Tea:	
1 tea bag/100 mL boiling water)	1/2000x
1.5 gram tea leaves/250mL boiling water	1/200x

If the concentration is determined to be out of standard range, adjust the dilution factor and make a new dilution. It is also possible to add a smaller volume of the diluted sample and make 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 µL of a secondary 1/10 dilution of a diluted sample, one may add 4.0 µL of the diluted sample and 36 µL of Sample Dilution Buffer). In the 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.

Assay Protocol:

For single tube luminometers or scintillation counters:

1. To a clean tube/vial used for luminescence measurement, add the following:
   A. Add 890 µL of Assay Buffer.
   B. Add 20 µL of Luminol Reagent, mix.
   C. Add 40 µL of Trolox Standard or sample, mix.

2. Incubate for several minutes if the temperature controller is used.

4. Add 50 µL of ABAP Reagent 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.

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Assay Protocol (cont.):

For luminescence plate readers:

1. Set measurement temperature slightly above (~0.2ºC) room temperature if the luminescence plate reader has a temperature controller. Otherwise, carry out experiment at room temperature.

2. Create standard/sample layout/template

3. Use a clean ***white*** 96-well plate. To each well used for test:
   A. Add 260 µL of Assay Buffer.
   B. Add 7 µL of Luminol Reagent
   C. Add 13 µL of Trolox Standard or sample, repeat withdrawing and pipetting a few times to mix.

4. Incubate for several minutes if temperature controller is used.

4. If the luminometer is equipped with an automatic injector, load ABAP solution (pool two vials of ABAP Reagent for whole plate assay) and then set-up to inject 16 µL of ABAP Reagent. Otherwise, use a multi-channel pipettor to add 16 µL ABAP Reagent to same row or same column simultaneously (use only limited (<4) rows or columns as the delay time is too high for a 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.

Data Analysis:

1. Using the RLU vs Time recording, plot the **induction time** (X-axis intercept of linear portion of the curve...see figure 1 page 10) vs concentration of Trolox Standards (x-axis). Perform a linear regression to get a standard curve as in Figure 1 page 10:

$$y = ax + b$$

2. The measured antioxidant concentration (in Trolox equivalents) of the samples in the assay [S_{assay}] is then:

$$[S_{assay}] = (Ts - b)/a$$

where Ts is the induction time of sample.

4. The antioxidant concentration (Trolox equivalent) in the original sample [$S_{original}$] is

$$[S_{original}] = [S_{assay}] * (\text{Dilution Factor})$$

Data Analysis (cont.):

Alternative Procedure and Calculation at Variable Temperature

With a single tube luminometer without a temperature control module and when laboratory temperature is deemed to fluctuate to a large degree experiments can be carried out with a single standard (e.g., 15 µM Trolox) and a sample (diluted) back-to-back. This procedure is also suitable for quickly estimating antioxidant status. 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 using a simple proportional equivalency ratio as follows:

$$[S_{assay}]/[C] = Ts/Tc$$

Where **[S_{assay}]** is the measured antioxidant capacity (in µM Trolox equivalents) of the diluted sample in the assay, **[C]** is the concentration of Trolox Standard tested and Ts and Tc are the induction times for Sample and Standard respectively. Simple rearrangement of the equation yields:

$$[S_{assay}] = (Ts/Tc) * [C]$$

Antioxidant Capacity of original sample is then...

$$[S_{original}] = [S_{assay}] * (\text{Dilution Factor})$$

Example Data for Standards and Samples:

The following examples show the expected behavior and results using the NWLSS™ TAC Peroxyl assay in determination of Antioxidant Capacity using the single tube luminometer method.

A typical standard curve of several Trolox standards is shown on the next page in Figure 1. Without a Trolox Standard (or sample antioxidant), the luminescence signal is very strong and decays slightly with time.

As can be seen, Trolox almost completely extinguishes this luminescence. When the Trolox or sample antioxidant is exhausted (**induction time**), luminescence rapidly increases to reach a plateau and then will decay slowly. The NWLSS™ TAC-Peroxyl Assay uses the x-axis intercept of the fast changing part of this time course curve to measure the induction time.