Start Here

About This Kit

Reactive oxygen species (ROS) are produced as a consequence of normal aerobic cell metabolism and as a response to exogenous factors including UV light, cigarette smoke, environmental pollutants, etc. Once formed, ROS easily react with other molecules within cells and cause damage to cellular and intracellular structures, including cell membranes, proteins, DNA and mitochondria. ROS are known to be one of the main causative factors involved in the pathogenesis of diverse diseases such as neurodegeneration, diabetes, cancer, rheumatoid arthritis, atherosclerosis, etc. Physiologically, there are complicated systems of defense against ROS-induced tissues damage which provided by macro and micro molecules and enzymes. Antioxidants act as a cooperative network, employing a series of redox reactions. Thus, quantitative measurement of the total antioxidant capacity (TAC) may provide more relevant biological information compared to that obtained by measurement of individual components.

TPR's TAC Assay Kit is a well-established tool for measuring TAC in serum, plasma, tissues, cell lysates and other biological fluids. This kit utilizes a peroxidase chromogenic substrate, which produces a water-soluble chromogen upon oxidation by ferryl myoglobin radicals. The green chromogen formation rate is inhibited by presence of antioxidants in environments and is measurable photometrically.

Kit Components

Item Label	Item	48 Tests Kit	96 Tests Kit
Reagent 1 (**R1**)	TAC Assay Buffer (10X)	5 ml	5 ml
Reagent 1 (**R2**)	TAC Assay Chromogen	Lyophilized	Lyophilized (2 vials)
Reagent 2 (**R3**)	TAC Assay Myoglobin	Lyophilized	Lyophilized
Reagent 4 (**R4**)	TAC Assay Standard	Lyophilized	Lyophilized
Reagent 5 (**R5**)	TAC Assay H_2O_2 (8.82 M)	200 µl	200 µl
-	96 Well Microplate	1 Plate	1 Plate
-	Technical Manual	1 Manual	1 Manual

Materials Required (Not Provided)

- Double distilled water (ddH2O).
- Disposables 15 ml and 1.5 ml tubes.
- Dounce homogenizer (if using tissue).
- Sonicator.

Required Instrumentation

- Multiwell microplate reader (capable of measuring absorbance at 412±7 nm or 734±16 nm).
- Microfuge
- Pipettes with variable volume setting.

Kit Performance

- **Precision:** Human TAC with 10 replicates on 3 different days showed the intra and inter assay coefficient of variation 5.7% and 3.7% respectively.
- **Range:** Samples containing antioxidants levels between 45-420 µM can be assayed without dilution.

Storage and Stability

- This kit will perform as specified if **stored at 4°C**.
- Use before the **expiration date** indicated on the box.

Sample Preparation

Important Note:

- All samples should be assayed immediately upon collection or stored at -80°C for up to 1-2 months.
- Always thaw samples on ice (Regulation of temperature at 4°C increases sensitivity and reproducibility).
- Reagents present in different extraction buffers may interfere with the assay, use 1X Assay buffer for extraction instead.

PLASMA SAMPLES

Typically, human plasma has an antioxidant capacity of 0.5-2 mM (Benzie et al. 1996, Kampa et al. 2002).

1. Collect whole blood using an anticoagulant such as heparin or citrate. Don't use EDTA.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C.
3. Carefully pipette off the plasma layer without disturbing the buffy layer, transfer to fresh tubes and place on ice. For long-term storage, store in working aliquots at –80 °C. The plasma samples will be stable for at least one month.

Note: Plasma should be diluted 20-30 times with 1X Assay Buffer prior to assay TAC.

SERUM

Typically, human serum has an antioxidant capacity of 0.5-2 mM (Miller et al. 1997, Koracevic et al. 2001).

1. Collect whole blood without using an anticoagulant.
2. Allow blood to clot for 20 minutes at 25°C.
3. Centrifuge the blood at 2000 x g for 15 minutes at 4°C.
4. Carefully pipette off the yellow serum layer without disturbing the buffy layer and transfer to a new tubes and place on ice. For long-term storage, store in aliquots at –80 °C. The serum samples will be stable for at least one month.

Note: Serum should be diluted 20-30 times with 1X Assay Buffer prior to assay TAC.

TISSUE HOMOGENATES

1. Weight out the amount of tissue necessary for each assay (~100 mg wet tissue).
2. Homogenize the tissue in 0.5 ml ice cold 1X Assay Buffer
3. Centrifuge at 12,000 x g for 15 minutes at 4°C.
4. Collect the supernatant and transfer to fresh tubes and place on ice. For long-term storage, store in working aliquots at –80 °C.

CELL LYSATES

1. Harvest the number of cells necessary for each assay (~1 x 10^6 cells/ml recommended). For harvesting adherent cells, don't use proteolytic enzymes (like trypsin); rather use a rubber policeman.
2. Lyse cells by repeated cycles of freezing and thawing or sonication in 1 ml ice cold 1X Assay Buffer.
3. Centrifuge at 12,000 x g for 15 minutes at 4°C.
4. Collect the supernatant and transfer to fresh tubes and place on ice. For long-term storage, store in working aliquots at –80 °C.

URINE SAMPLES

Typically, human urine has an antioxidant capacity of 0.2-3 mM (Koracevic et al. 2001).
Collect urine in a clean container and place on ice. For long-term storage, store in aliquots at –80 °C.

Note: Urine should be diluted 10-20 times with Assay Buffer prior to assay TAC.

SALIVA SAMPLES

Typically, human saliva has an antioxidant capacity of 0.3-1 mM (Koracevic et al. 2001).
Collect saliva in a clean container and place on ice. For long-term storage, store in aliquots at –80 °C.

Note: Saliva should be diluted 2 times with 1X Assay Buffer prior to assay TAC.

Reagent Preparation

1) **1X Assay Buffer**: Dilute **R1** (10X Assay Buffer) ten-fold with ddH2O (e.g., add 2 ml of 10X Assay Buffer to 18 ml of ddH2O) and mix well. This 1X Assay Buffer is stable for six months if stored at 4°C.
2) **R2 Ready Reagent: R2** vial contains lyophilized powder of chromogen. Spin **R2** vial for 30 seconds. Add 1 mL of 1X Assay Buffer to vials and vortex well. Then transfer contents of vials to a tube already containing 7 mL 1X Assay Buffer and vortex well. One reconstituted vial is sufficient for 48 wells. *R2 Ready Reagent* is stable for 24 hours at 4°C.
3) **R3 Ready Reagent: R3** vial contains lyophilized powder of Myoglobin. Spin **R3** vial for 30 seconds. Add 600 µL of 1X Assay Buffer to vial (48 Tests Kit)/Add 1.2 mL of 1X Assay Buffer to vial (96 Tests kit) and vortex well. Store in working aliquots at –20 °C. The *R3 Ready Reagent* remains active for one month at –20 °C.
4) **R4 Ready Reagent: R4** vial contains lyophilized powder of TAC Standard (TROLOX). Add 2.67 mL of 1X Assay Buffer to the vial and vortex well until totally dissolved. This reconstituted *Standard stock solution* is used to prepare the standard curve. Store *Standard stock solution* in working aliquots at –20 °C. This stock is stable for 24 hours at 4 °C and for 3 months at -20 °C.
5) **R5 Ready Reagent:** This vial contains an 8.82 M solution of hydrogen peroxide. Dilute 10 µL of **R5** reagent with 990 µL of ddH2O. Further dilute by removing 20 µL and diluting with 3980 µL of ddH2O to prepare *R5 Ready Reagent*. This reagent is stable for 4 hours at room temperature.

Standard Preparation

Take six clean microtubes and label them A-F. Add the amount of *standard stock solution* and 1X Assay Buffer to each tube as described in below.

Tubes	Standard Stock Solution (µL)	1X Assay Buffer (µL)	Final Concentration (µM)
A	0	500	0
B	5	495	15
C	15	485	45
D	35	465	105
E	70	430	210
F	140	360	420

Things to Note

- Except samples, allow all reagents to equilibrate to room temperature before performing the assay.
- It is recommended that all samples and standards be assayed at least in duplicate.
- The final volume in each well is 210 µL.

Assay Protocol

1. Shake all samples for homogenation.
2. Add 10 µL standards/samples to related wells.
3. Add 150 µL **R2 Ready Reagent** to all wells.
4. Add 10 µL **R3 Ready Reagent** to all wells and incubate for 5 minutes.
5. Add 40 µL **R5 Ready Reagent** to all wells to initiate the reaction. Perform this step as quickly as possible (*within one minute is recommended / Multi-channel pipettes are preferred at this step*).
6. Cover the plate with plate cover and incubate on shaker at room temperature for 5 minutes.
   Note: The five minutes' incubation is suggested as a guideline. If required, the incubation time can be changed (increased or decreased) in order to obtain a measurable absorbance.
7. Read absorbance at 734±16 nm or 412±7 nm using a plate reader.

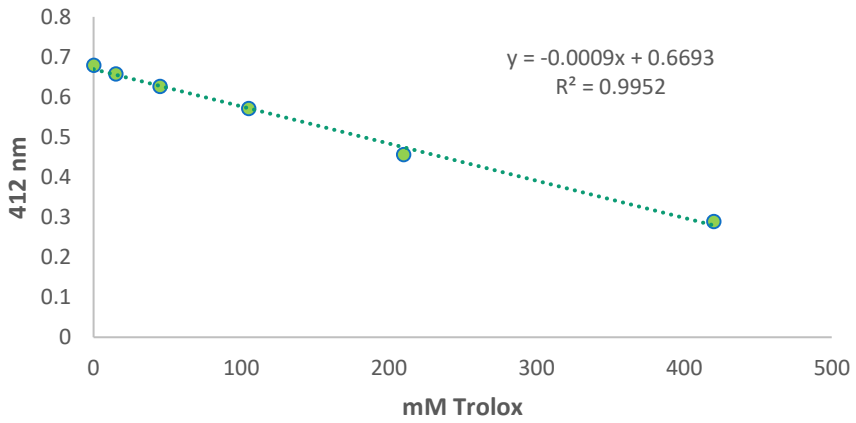
Calculations

1. Calculate the average absorbance of the wells for each standard and sample.
2. Plot the average absorbance values of each standard as a function of the final standard concentration (µM).
3. Calculate the antioxidant concentration for each sample using the equation obtained from the standard curve.

$$\text{TAC (µM)} = \frac{(Sample\ average\ absorbance\ ) - (\text{y-intercept})}{Slope}\ \text{X}\ dilution\ factor$$

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Absorbance <0.05 in sample wells	1. High concentration of antioxidants 2. Interference reagent in samples	Dilute samples with 1X assay buffer and re-assay
No antioxidants were detected in samples	Sample was too dilute	Re-assay with lower dilutions
Erratic values; dispersion of duplicate/triplicates	1. Poor pipetting/technique 2. Bubble in wells	1. Be careful not to splash the contents of the wells 2. Remove bubbles by tapping the side of plate



TROLOX Final Conc.	Absorbance
0	**0.679**
15	**0.657**
45	**0.626**
105	**0.571**
210	**0.456**
420	**0.289**

Fig. 1: Typical standard curve