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Product Information

Antioxidant Assay Kit

Catalog Number **CS0790**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

Free radicals or reactive oxygen species (ROS) are produced during biochemical redox reactions as part of normal physiological cell metabolism (protection from infectious organisms) and as a response to environmental factors such as UV light, cigarette smoke, environmental pollutants, and γ-radiation. Once formed, ROS attack cellular components causing damage to lipids, proteins, and DNA, which can initiate numerous diseases, including cancer, atherosclerosis, rheumatoid arthritis, diabetes, liver damage, and central nervous system disorders.

Living organisms have a large number of antioxidants, including macro and micro molecules, and enzymes, which represent the total antioxidant activity of the system, and play a central role in preventing oxidative stress. Therefore, quantitative measurement of the cumulative antioxidant capacity of body fluids, tissues, and cells, following different stimuli, may provide important biological information.

The principle of the antioxidant assay is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, $ABTS^{\bullet+}$, a soluble chromogen that is green in color and can be determined spectrophotometrically at 405 nm.

$$HX\text{-}Fe^{III} + H_2O_2 \rightarrow {}^{\bullet}X\text{-}[Fe^{IV}{=}O] + H_2O$$

$$ABTS + {}^{\bullet}X\text{-}[Fe^{IV}{=}O] \rightarrow ABTS^{\bullet+} + HX\text{-}Fe^{III}$$

In this equation, $HX\text{-}Fe^{III}$ is metmyoglobin and ${}^{\bullet}X\text{-}[Fe^{IV}{=}O]$ is ferryl myoglobin.

Antioxidants suppress the production of the radical cation in a concentration dependent manner and the color intensity decreases proportionally. Trolox™, a water-soluble vitamin E analog, serves as a standard or control antioxidant.

The kit provides all of the reagents required for an efficient measurement of the total antioxidant capacity of plasma, serum, urine, saliva, cells, and tissue lysates. It was tested on A431 and CHO cell lysates; rat brain, liver, and kidney lysates; human plasma, serum, urine, and saliva.

Components

The kit is sufficient for 200 assays in 96 well plates.

Assay buffer, 10× Catalog Number A3605	30 ml
Stop Solution Catalog Number S3446	20 ml
Myoglobin from horse heart Catalog Number M1882	2 × 1 mg
(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) Catalog Number 238813	2 × 1 mg
ABTS, 10 mg tablets Catalog Number A9941	4 tablets
Phosphate-Citrate Buffer, pH 5 Catalog Number P4809	4 tablets
Hydrogen Peroxide, 3% solution Catalog Number 323381	1 ml

Equipment Required but Not Provided

96 well plates, flat bottom (Catalog Number P7366 or equivalent)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Use ultrapure water to prepare all solutions.

1× Assay Buffer - Dilute the Assay Buffer, 10× (Catalog Number A3605) ten-fold with ultrapure water (*e.g.*, add 2 ml of Assay Buffer, 10× to 18 ml of ultrapure water) and mix well.

Myoglobin Solutions
Note: The myoglobin (Catalog Number M1882) is used as the source of metmyoglobin in the assay reaction.

1. Myoglobin Stock Solution - Reconstitute the Myoglobin (Catalog Number M1882) by adding 285 µl of ultrapure water to the vial and vortexing well. Store in working aliquots at –20 °C. The Myoglobin Stock Solution remains active for 6 months at –20 °C.
2. Myoglobin Working Solution - Before use, dilute the required amount of Myoglobin Stock Solution 100-fold with 1× Assay Buffer and mix well. For each well of Trolox Standard or Test sample, prepare 20 µl of the Myoglobin Working solution.

Trolox Working Solution - Reconstitute the Trolox (Catalog Number 238813) by adding 2.67 ml of 1× Assay Buffer and vortexing until totally dissolved. This reconstituted 1.5 mM Trolox Working Solution is used to prepare the Trolox standard curve. The solution can be stored in working aliquots at –20 °C for at least three months.

ABTS Substrate Solution – Add one ABTS tablet (Catalog Number A9941) and one Phosphate-Citrate Buffer tablet (Catalog Number P4809) to 100 ml of ultrapure water and mix until totally dissolved. This solution can be stored at 4 °C for no more then two weeks or at –20 °C for at least one month.

Test Sample – See Appendix for sample preparation guidelines. Concentrated samples (*e.g*. serum, plasma, and lysates) should be diluted with the 1× Assay Buffer.

Storage/Stability
The kit is shipped on wet ice and storage at 2–8 °C is recommended. Upon first use the Phosphate-Citrate Buffer tablets (Catalog Number P4809), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Catalog Number 238813), ABTS tablets (Catalog Number A9941), and Stop Solution (Catalog Number S3446) can be stored at room temperature.

Procedure
Perform assays in duplicate and use ultrapure water.
Note: Antioxidant levels of the Test Samples should fall within the range of the standard curve. Samples containing antioxidant levels between 0.015–0.42 mM (Trolox equivalents) can be tested without dilution or concentration. When necessary, the Test Sample can be diluted with 1× Assay Buffer prior to assay to bring the antioxidant level within range.

1. Prepare Trolox Standards for a standard curve according to Table 1.

Table 1.
Trolox Standards

Tube	1.5 mM Trolox Working Solution (µl)	1× Assay Buffer (µl)	Trolox concentration in the standard (mM)
1	0	500	0
2	5	495	0.015
3	15	485	0.045
4	35	465	0.105
5	70	430	0.21
6	140	360	0.42

2. Prepare ABTS Substrate Working Solution by adding 25 µl of 3% Hydrogen Peroxide Solution (Catalog Number 323381) to 10 ml of ABTS Substrate Solution (Preparation Section). Use within 20–30 minutes.
3. Prepare assays in the 96 well plate.
   - In wells for the Trolox standard curve, add 10 µl of a Trolox Standard (from tubes 1-6) and 20 µl of Myoglobin Working Solution.
   - In wells for the Test Samples, add 10 µl of Test Sample and 20 µl of Myoglobin Working Solution.
4. Add 150 µl of ABTS Substrate Working Solution (step 2) to each well.
5. Incubate for 5 minutes at room temperature.
   Note: The five minute incubation is suggested as a guideline. If required, the incubation time can be changed (increased or decreased) in order to obtain a measurable absorbance.
6. Add 100 µl of Stop Solution (Catalog Number S3446) to each well. Prior to use, warm the Stop Solution to room temperature and mix until homogeneous.
7. Read the endpoint absorbance at 405 nm using a plate reader.
   Note: The plate should be read within an hour.

Results

Calculation

1. Calculate the average absorbance of the wells for each Trolox Standard.
2. Prepare a standard curve by plotting the average absorbance of each Trolox Standard as a function of the final Trolox concentration (mM).
3. Calculate the antioxidant concentration of the Test Sample using the equation obtained from the linear regression of the standard curve (see Figure 1 for standard curve example).

$$X\ (mM) = \frac{y(A_{405}) - \text{Intercept}}{\text{Slope}} \times \text{dilution factor}$$

X (mM) - Antioxidant concentration [(mM) relative to the concentration of the Trolox standard].

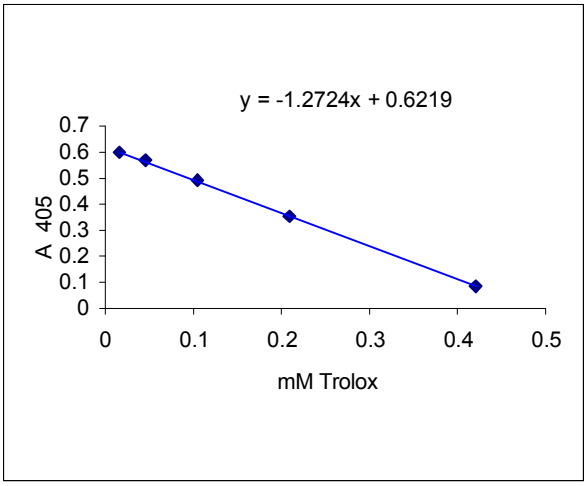
$y(A_{405})$ - the average absorbance of the Test Sample at 405 nm

Intercept - intercept of the Y axis by the standard curve (0.6219 in Figure 1)

Slope - Slope of the standard curve, a negative value (–1.2724 in Figure 1)

dilution factor - fold dilution of the original sample (will be used only if sample was diluted prior to adding to the well)

Figure 1.
Example of Trolox Standard Curve



References

1. Miller, N.J., and Rice-Evans, C.A., Factors influencing the antioxidants activity determined by the $ABTS^{+}$ radical cation assay. Free Radic. Res., **26**, 195-199 (1997).
2. Proteggente, A.R., *et al*., Gender differences in steady-state levels of oxidative damage to DNA in healthy individuals. Free Radic. Res., **36**, 157-162 (2002).
3. Huang, H., *et al*., The chemistry behind antioxidant capacity assays. J. Agric. Food Chem., **53**, 1841-1856 (2005).
4. Rice-Evans, C.A., Measurement of total antioxidant activity as a marker of antioxidant status *in vivo*: procedures and limitations. Free Radic. Res., **33** Suppl, S59-66 (2000).

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Appendix

Sample preparation

Different reagents present in the extraction buffer may interfere with the assay. If using an extraction buffer other then the 1× Assay Buffer, it is important to verify that the buffer components do not affect the results.

Detergents, such as TWEEN® 20, TRITON® X-100, and IGEPAL® CA-630 (Nonidet P-40), should not be present at any concentration in the test sample. CHAPS can be present in the sample at a final concentration ≤0.2%.

Reducing materials like DTT and 2-mercaptoethanol may also interfere with the assay.

A. Cell Culture Lysate:
Collect and centrifuge ~1 × 10^6 cells. Homogenize or sonicate the pellet on ice in 0.5–1 ml of cold 1× Assay Buffer and then centrifuge at 12,000 × *g* for 15 minutes at 4 °C. Remove the supernatant and keep it on ice. For long-term storage, store in working aliquots at –70 °C.

B. Tissue Lysate:
Homogenize tissue samples on ice in cold 1× Assay Buffer (~100 mg of tissue/0.5 ml of buffer) and then centrifuge at 12,000 × *g* for 15 minutes at 4 °C. Remove the supernatant and keep it on ice. For long-term storage, store in working aliquots at –70 °C.

C. Plasma:
Collect blood with anticoagulant (heparin or sodium citrate) and centrifuge at 1,000 × *g* for 10 minutes at 4 °C. Remove the supernatant and keep it on ice. For long-term storage, store in working aliquots at –70 °C.

D. Serum:
Collect blood without anticoagulant, allow blood to clot, and centrifuge at 2,000 × *g* for 10 minutes at 4 °C. Remove the supernatant and keep it on ice. For long-term storage, store in working aliquots at –70 °C.