Cu-TAC Antioxidant Assay Kit
Cat# AOX-4

INSTRUCTION MANUAL ZBM0088.00

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier at 4°C.
All orders must be processed immediately upon arrival.

Cu(II)Cl_2, Neocuproine and Assay Buffer Solutions

Store at 4°C.

Trolox and Uric acid standards

Store at -20°C

Long-term storage:

Remove the Cu(II)Cl2, Neocuproine and Assay Buffer Solutions from the box and place at 4°C, store the Trolox and Uric acid standard solutions at -20°C. Reagents are good for at least 3 months after arrival if stored properly.

For *in vitro* Use Only

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress, however, excess ROS can overwhelm the systems and cause severe damage.

The Zen-Bio Cu-TAC Antioxidant Assay Kit can be used to determine the total antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The assay measures the reduction of Copper(II) to Copper(I) in the presence of the aromatic chelator, neocuproine. Uric acid or Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control for the reduction reaction in a dose dependent manner. The antioxidant activity in biological fluids, cells, tissues, and natural extracts can be normalized to equivalent Uric acid or Trolox units to quantify the composite antioxidant activity present.

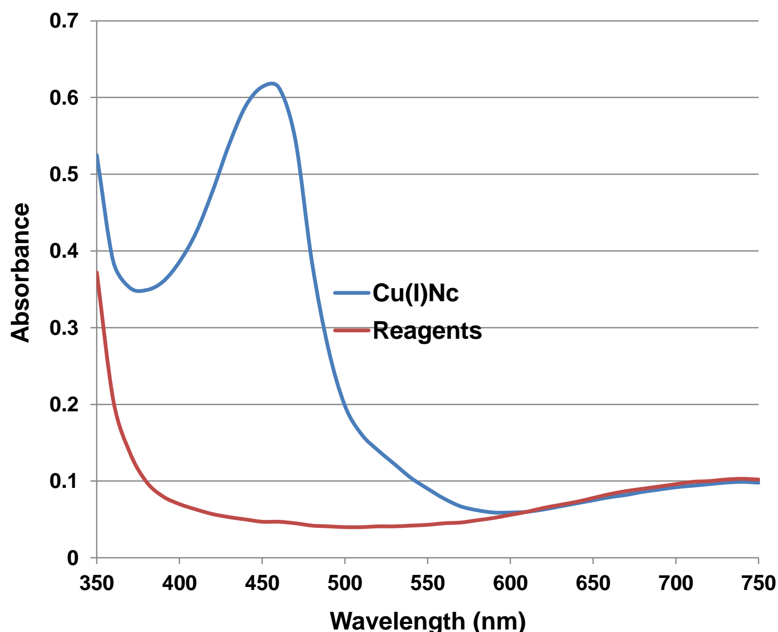
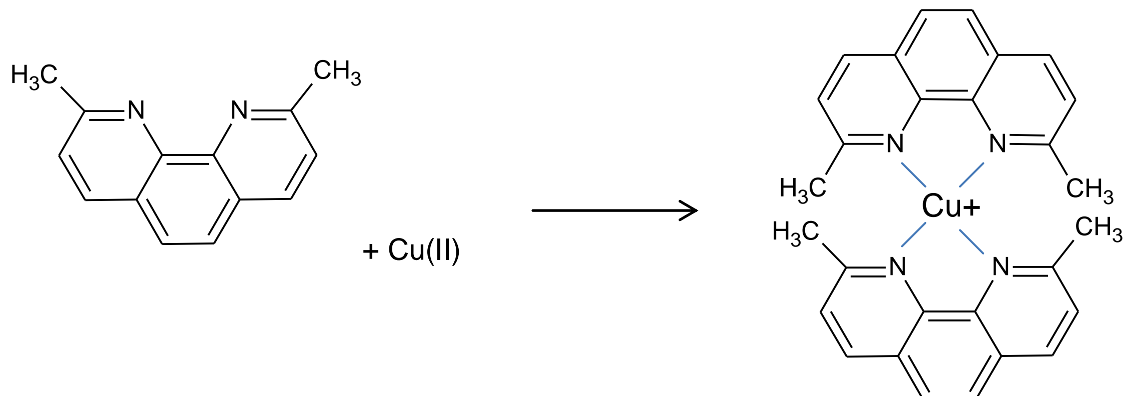


Figure 1. Cu(I) neocuproine (Cu(I)Nc) complex absorbance spectrum

PRINCIPLE OF THE ASSAY

Copper (II) can be reduced to copper (I) by antioxidants, such as trolox or uric acid, through an electron transfer mechanism. The Cu(I) is stabilized by the neocuproine in a complex that blocks its oxidation back to Cu(II). This Cu(I)-neocuproine complex produces a color that has an absorbance maximum at 450 nm. By comparing the 450 nm absorbance induced by test samples to that induced by the trolox or uric acid standard curve, the relative antioxidant activity of test samples can be determined.



ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Color	UNIT	QTY	STORAGE
Blank Assay Plate	96-well assay plate, blank	---	PLATE	1	-----
AOX4 Assay Buffer	6 ml	CLEAR	BOTTLE	1	4°C
Cu(II)Cl_2 solution	6 ml	CLEAR	BOTTLE	1	4°C
Neocuproine Solution	6 ml	CLEAR	BOTTLE	1	4°C
AOX4 Trolox	5mM	AMBER	50 µl /VIAL	1	-20°C
AOX4 Uric Acid	1mM	AMBER	250 µl /VIAL	1	-20°C
Tray	For multi-channel pipetters	---	EACH	2	RT

Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Water
- Plate reader with a filter of 450 nm, or within a range of 425 – 470 nm (See figure 1).

Reagents that might interfere with the assay results:

EDTA
> 1% TWEEN 20
> 1% TRITON X-100
IGEPAL CA-630 (Nonidet P-40)
> 0.2% CHAPS
EGTA and other chelators
2-mercaptoethanol
Tris
Borate
DTT

SAMPLE PREPARATION

Cell Lysate Preparation

1. Scrape ~1 $x10^6$ cells and centrifuge at 1,000xg to prepare a cell pellet. DO NOT use proteolytic enzymes such as trypsin but scrape using a rubber policeman or cell scraper tool.
2. Homogenize or sonicate the cell pellet on ice in 1ml cold PBS
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and keep on ice until ready to use in the assay.
5. If not using the same day, store the samples at -80°C.
6. Data is expressed as Trolox or Uric Acid equivalents (TE or UAE) per cell number (i.e. µmole TE/10^6 cells)

Tissue Lysate Preparation

1. Homogenize tissue samples on ice in cold PBS at ~200mg tissue per ml cold buffer
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as Trolox or Uric Acid equivalents (TE or UAE) per gram of starting sample (i.e. µM TE/g)

Plasma Preparation

1. Collect the blood in a tube containing heparin **[NOT EDTA]**.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox or Uric Acid equivalents (TE or UAE) per volume sample (i.e. µmole TE/L)

Serum Preparation

1. Collect the blood in a tube WITHOUT any anticoagulant. Allow the blood to clot.
2. Centrifuge at 2,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox or Uric Acid equivalents (TE or UAE) per volume sample (i.e. µmole TE/L)

Urine Preparation

1. Collect urine and test undiluted or diluted with PBS.

Food Extract Preparation

Weigh the starting material.

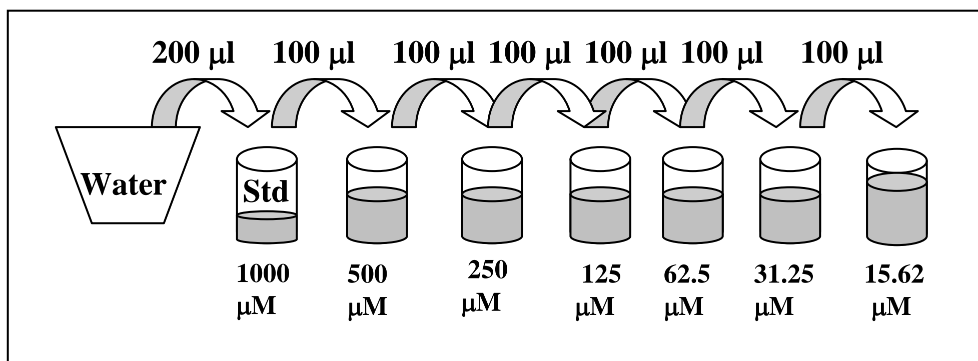
1. Homogenize in a small volume ice cold PBS or water.
2. Store small aliquots at -80°C for analysis.
3. When ready to assay, keep thawed samples on ice.
4. Data is expressed as Trolox or Uric Acid equivalents (TE or UAE) per gram of starting sample (i.e. µM TE/g)

ASSAY PROCEDURE

1. Remove reagents from the refrigerator and allow them to come to room temperature.

2. Prepare standards as follows (either Trolox or Uric acid may be used):

   Trolox: Briefly spin down the contents of the 5 mM Trolox standard tube after thawing. Pipette 200 µl of water into the 5 mM Trolox standard tube provided and mix well by vortexing. This produces a diluted stock Trolox standard of 1000 µM. Pipette 100 µl of water into 6 tubes (not provided). Using the newly diluted stock Trolox solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 1000 µM stock dilution serves as the highest standard, and water serves as the zero standard.



   Uric Acid: Briefly spin down the contents of the 1000 µM Uric Acid standard tube after thawing. Pipette 100 µl of water into 6 tubes (not provided). Using the stock 1000 µM Uric acid solution, prepare a dilution series as depicted above. Mix each new dilution thoroughly before proceeding to the next. The 1000 µM stock dilution serves as the highest standard, and water serves as the zero standard

3. Add 50 µl of Neocuproine solution and 50 µl of Assay buffer to each well.

4. Add 20 µl of the diluted standards or samples to the appropriate wells.

5. Read absorbance at 450 nm to determine intrinsic absorbance of your samples.

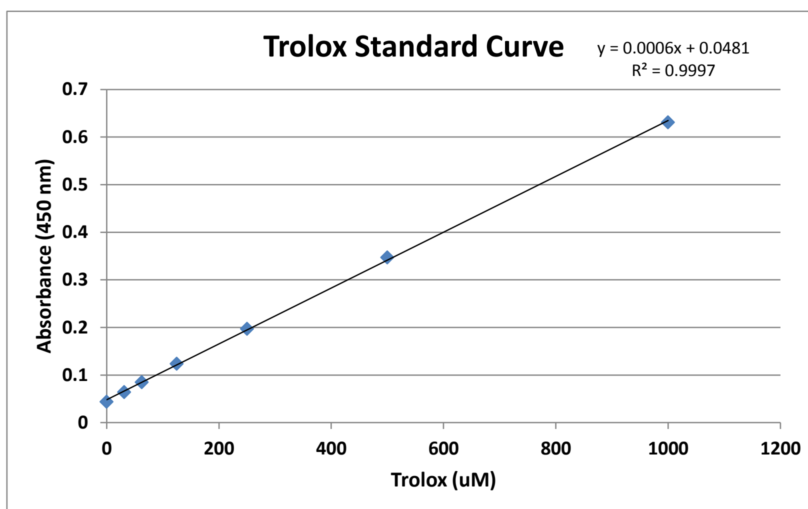
6. To begin the assay, add 50 µl of the Cu(II)Cl_2 solution per well and place at 37 °C. Allow the reaction to proceed for 30 minutes. **[The trolox reaction is completed within 5 minutes, uric acid and other antioxidants may require up to 30 minutes for assay completion].**

7. Read absorbance using plate reader at a wavelength of 450 nm.

STANDARD CURVE ___________________________

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

uM Trolox	Avg Abs	Stdev
1000	0.631	0.011314
500	0.347	0.005657
250	0.197	0.001414
125	0.1235	0.000707
62.5	0.085	0
31.25	0.064	0.001414
0	0.044	0.001414



y = observed O.D.
x = concentration of Trolox in μM
To calculate x for each y, (i.e. to change the observed O.D. into Trolox equivalent concentration) use the following equation:
y=(slope) times (x) plus intercept
y=mx+b so x=(y-b)/m
x=(y – 0.0481)/(0.0006) where (0.0006)= slope of the line and 0.0481= y intercept. Be careful to enter the proper sign for the y intercept value as it may be a negative number.

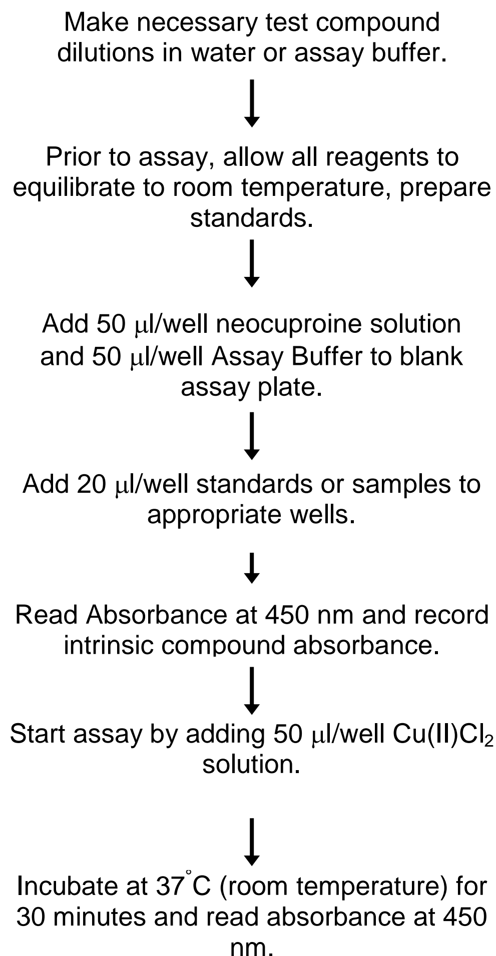
The R^2 value should be equal or greater than 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

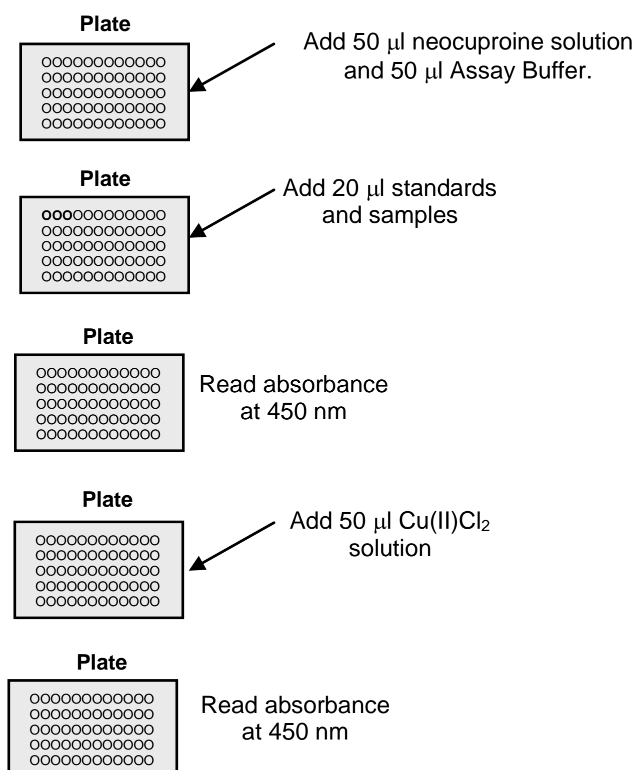
Antioxidant activity is expressed as μM Trolox equivalents (TE) or Uric acid equivalents (UAE) if it was used as the standard.

APPENDIX A: Plate layout ______________________________

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1000 µM	Standard 1000 µM										
B	Standard 500 µM	Standard 500 µM										
C	Standard 250 µM	Standard 250 µM										
D	Standard 125 µM	Standard 125 µM										
E	Standard 62.5 µM	Standard 62.5 µM										
F	Standard 31.25 µM	Standard 31.25 µM										
G	Standard 15.62 µM	Standard 15.62 µM										
H	Standard 0 µM	Standard 0 µM										

APPENDIX B: Protocol Flowchart





ZenBio Cu-TAC and TAC-BCS assays:
Both assays monitor the reduction of Cu(II) to Cu(I) which is stabilized by an aromatic chelator. The Cu-TAC reaction is slower and requires higher temperature; however, it is capable of assessing lipophilic antioxidants in plasma, tissue homogenates and other extracts. The TAC-BCS assay is rapid and works quite well with aqueous antioxidants and water soluble components of biological fluids and extracts.

REFERENCES

1. Free Radical Res., 39: 949, 2005.
2. Analytical Sciences, 27: 483-488, May 2011.
3. Microchim. Acta, 160: 413, 2008.