Cupric Ion Chelating (CIC) Assay Kit

Cat# AOX-16

INSTRUCTION MANUAL ZBM0125.00

STORAGE CONDITIONS

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

Pyrocatechol Violet Solution

Remove from box and store at -20°C

All Other Reagents and Assay Plates

Store at room temperature

Long-term storage:

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.

Excess transition metal ions, such as Cu(II), can generate hydroxyl radicals [OH•] in biological systems through Fenton-like reactions. Some antioxidants are able to chelate Cu(II) thereby inhibiting the formation of hydroxyl radicals and oxidative damage. The ZenBio Cupric Ion Chelating (CIC) Assay measures the capacity of test samples to chelate free cupric ions in solution thereby inhibiting Cu(II) binding to pyrocatechol violet (PV) which generates a highly colored complex. EDTA serves as a positive control capable of chelating cupric ions in a dose dependent manner. The CIC assay is an endpoint assay measuring absorbance of the cupric-PV complex at λ = 632nm. CIC activity is determined as the percent of total PV / Cu(II) binding.

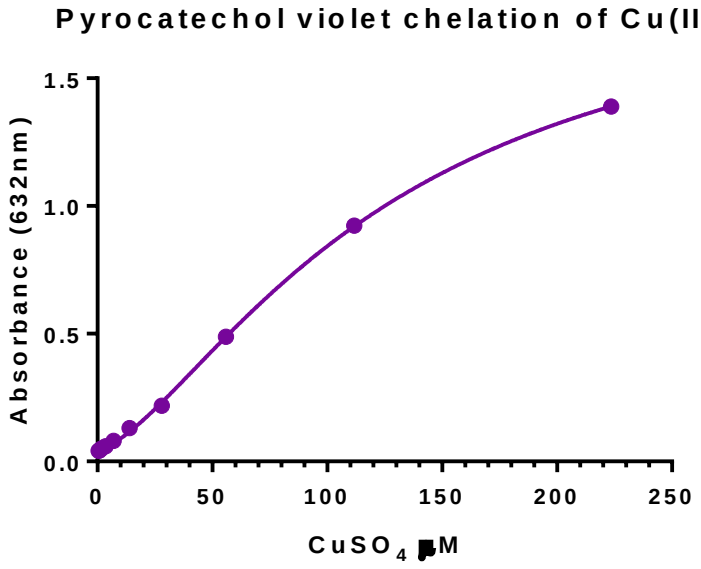


Figure 1. Cu(II) Chelating Activity of PV

Pyrocatechol Violet (2mM) chelates Cu(II) to produce a highly colored complex. Absorbance at 632nm increases with increasing Cu(II) concentration in solution.

PRINCIPLE OF THE ASSAY

Pyrocatechol Violet (Pyrocatecholsulfonphthalein, PV) is able to form a complex with free cupric ions (Cu(II)) resulting in a chromophore with strong absorbance at 632nm. Compounds that are able to chelate Cu(II) lower the amount of free Cu(II) in solution, decreasing the PV-Cu(II) complex concentration, which results in a loss of absorbance at 632nm.

Cu(II) + Pyrocatechol Violet (PV) ⟶ PV * Cu(II) (highly colored)

Cu(II) + Chelator + PV ⟶ Chelator*Cu(II) + PV (less color)
[Cu(II) chelators reduce PV-Cu(II) formation which generates less color]

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Color	UNIT	QTY	STORAGE
Blank Assay Plates	96-well assay plates, blank	---	PLATE	2	-----
AOX Assay Buffer	50 ml	CLEAR	BOTTLE	1	RT
$CuSO_4$ Solution	100 mg/mL (1000x)	AMBER	50 µl /VIAL	1	RT
Pyrocatechol Violet (PV) Solution	2mM	AMBER	VIAL	2	-20°C
EDTA solution	1mM in Assay Buffer	CLEAR	1 mL /VIAL	1	RT
Tray	For multi-channel pipetters, clear polyvinyl	---	EACH	2	RT

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

- Multi-channel Pipet , single channel pipet and pipet tips
- Tubes for preparing dilutions.
- Distilled water
- Plate reader with a filter of ~632 nm

Reagents that might interfere with the assay results:
EDTA and other strong iron chelators

ASSAY PROCEDURE

1. Prepare 1x $CuSO_4$ Solution by adding 5 µL 1000x stock to a tube containing 5 mL of distilled water. Mix by vortexing. 3.5 mL is needed per plate.
2. Thaw Pyrocatechol Violet (PV) solution to room temperature (RT) prior to performing the assay. Mix by vortexing.
3. Prepare test sample dilutions in Assay Buffer as necessary.
4. Add 30 µL of Assay Buffer to 6 wells. Three wells are for background absorbance determination and three wells are for maximal PV chelating values.
5. Add 30 µL of samples and 1mM EDTA to triplicate wells.
6. Add 200 µL of Assay Buffer to all of the plate wells.
7. Add 30 µL of distilled water to the three wells for background determination from step 4, and then add 30 µL of the 1x $CuSO_4$ Solution to all of the remaining wells.
8. Incubate at RT for 5 minutes.
9. To begin the assay, add 8.5 µL of the Pyrocatechol Violet (PV) Solution to each well of the assay plate and incubate, shaking at room temperature for 10 minutes. Remove from shaker and incubate an additional 10 minutes at room temperature.
10. Determine absorbance using plate reader at a wavelength of 632 nm.

ALTERNATIVE ASSAY PROCEDURE

This includes an EDTA standard curve to determine EDTA equivalents of test samples.

Prepare the EDTA standards according to the following table:

Standard	Final Conc. (µM)	Assay Buffer (µL)	Volume (µL)	EDTA Solution
Std1000	1000	0	300	1000 µM solution
Std500	500	150	150	Std1000
Std250	250	150	150	Std500
Std125	125	150	150	Std250
Std62.5	62.5	150	150	Std125
Std31.25	31.25	150	150	Std62.5
Std15.6	15.6	150	150	Std31.25
Std0	0	150	0	0

Mix each new dilution thoroughly before proceeding to the next step.

Add 30 µL of each standard to duplicate wells on each assay plate used.

DATA ANALYSIS

Values are determined as a percent Cupric ion chelating (%).

1. Determine the background absorbance value by averaging the absorbance values of the three background wells (no $CuSO_4$ or sample).
2. Subtract the average background absorbance from all of the absorbance values.
3. Determine the maximal absorbance value by averaging the absorbance values of the three wells containing $CuSO_4$, PV and Assay Buffer (from step 4, above).
4. Use the following equation to determine the cupric ion chelating percent:

   Cupric ion chelating (%) = 100 x $(Abs_{max} - Abs_{test})/Abs_{max}$

   Abs_{max} is determined in step 3.

   Abs_{test} is the absorbance of the test sample.

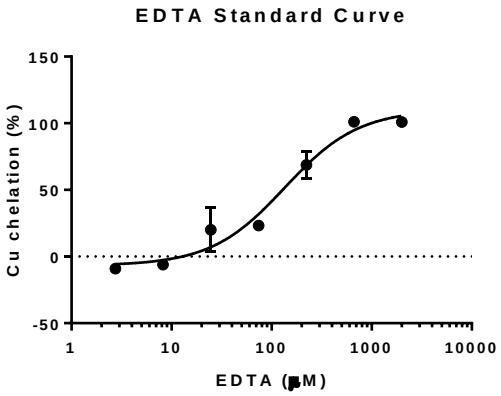
ALTERNATIVE DATA ANALYSIS

Values are determined as µM EDTA equivalents.

1. Determine Cupric ion chelating (%) for al controls and samples as described above.
2. Plot EDTA concentration vs. Cupric ion chelating (%).

EDTA uM	Chelating Activity (%)		Avg.	Stdev
2000	100	101	100	0.53
667	100	101	101	0.12
222	75.8	61.8	68.8	9.93
74	22.0	24.5	23.2	1.77
24.7	31.9	8.43	20.2	16.6
8.23	-4.29	-7.82	-6.1	2.49
2.74	-7.34	-10.9	-9.1	2.49

This curve generated by 3-fold dilutions.



A three or four parameter fit may be applied to the data after log10 transforming the concentrations.

Use the values generated from your standard curve to back-calculate the equivalent EDTA concentration for each of your samples.

APPENDIX A: Plate layout _____

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX B: Protocol Flowchart

CUPRIC ION CHELATING ASSAY

Prior to assay, prepare 1x solution of $CuSO_4$ and bring PV solution to room temperature. Prepare EDTA standard curve if necessary.

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Make necessary test compound dilutions in Assay Buffer.

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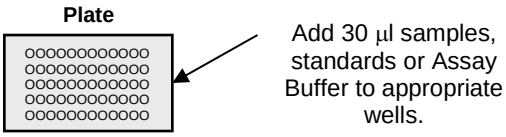
Add 30 µl/well samples or standards to assay plate. Add 30 µl/well Assay Buffer to background wells and maximum signal wells.



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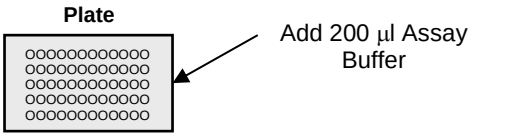
Add 200 µl/well Assay Buffer to all the wells of the plate.



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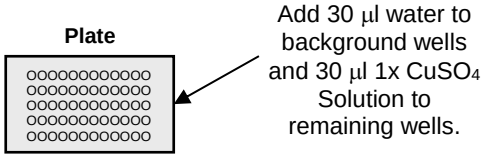
Add 30 µl distilled water to triplicate wells for background. Add 30 µl/well 1x $CuSO_4$ Solution to remaining wells. Incubate at RT for 5 minutes.



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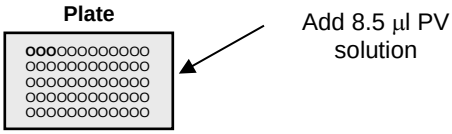
Start the assay by adding 8.5 µl/well PV Solution.



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Incubate shaking at 25˚C (room temperature) for 10 minutes. Turn off shaker and incubate an additional 10 minutes at 25˚C

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Measure the optical density of each well at 632 nm using a spectrophotometer plate reader.

REFERENCES

1. Nutrients, **11**(9):2007, 2019.
2. Food Chem., **214**:515-522, 2017

FREQUENTLY ASKED QUESTIONS _____

1. **Is it alright that my absorbance values are lower than those in the sample data?** Yes, the absorbance values may vary from different plate readers, however the background and maximum control values should be at least 0.2 absorbance units different.

2. **Should I dilute my sample for testing its CIC activity**? In order to accurately determine the CIC activity of your sample, it is likely necessary to prepare several dilutions to ensure that you generate a dose-dependent response.