Ferrous Iron Chelating (FIC) Assay Kit

Cat# AOX-15

INSTRUCTION MANUAL ZBM0124.01

STORAGE CONDITIONS

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

All Reagents and Assay Plate

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 Fe(II), can generate hydroxyl radicals [OH•] in biological systems through Fenton-like reactions. Some antioxidants are able to chelate Fe(II) thereby inhibiting the formation of hydroxyl radicals and oxidative damage. The ZenBio Ferrous Ion Chelating (FIC) Assay measures the capacity of test samples to chelate free ferrous ions in solution thereby inhibiting Fe(II) binding to ferrozine which generates a highly colored complex. EDTA serves as a positive control capable of chelating ferrous ions in a dose dependent manner. The FIC assay is an endpoint assay measuring absorbance of the ferrous-ferrozine complex at λ = 562nm. FIC activity is determined as the percent of total ferrozine / Fe(II) binding.

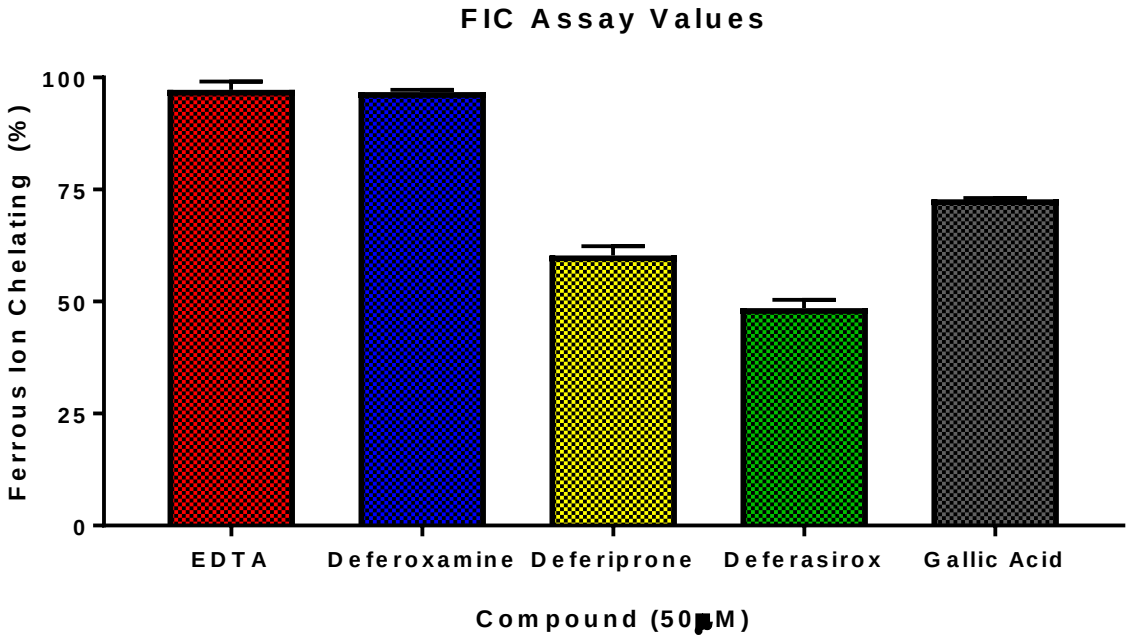


Figure 1. Fe(II) Chelating Activity of Compounds in the FIC Assay

EDTA, Deferoxamine, Deferiprone, Deferasirox, and Gallic acid were tested at 50µM for their Fe(II) chelation activity in the FIC assay.

PRINCIPLE OF THE ASSAY

Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazinep,p′-disulfonic acid) is able to form a complex with free ferrous ions (Fe(II)) resulting in a chromophore with strong absorbance at 562nm. Compounds that are able to chelate Fe(II) lower the amount of free Fe(II) in solution, decreasing the Ferrozine-Fe(II) complex concentration, which results in a loss of absorbance at 562nm.

Fe(II) + Ferrozine ⟶ Ferrozine * Fe(II) (highly colored)

Fe(II) + Chelator + Ferrozine ⟶ Chelator*Fe(II) + Ferrozine (less color)

[Fe(II) chelators reduce Ferrozine-Fe(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	30 ml	CLEAR	BOTTLE	1	RT
$FeSO_4$ powder	5 mg	AMBER	VIAL	1	RT
Ferrozine powder	5 mg	CLEAR	BOTTLE	1	RT
EDTA solution	50mM in Assay Buffer	CLEAR	50 µl /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 ~562 nm

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

ASSAY PROCEDURE

1. Prepare 100x $FeSO_4$ Solution by adding 1.8mL of distilled water to the tube containing 5mg of $FeSO_4$. Mix by vortexing.
2. Prepare 100x Ferrozine Solution by adding 406µL of distilled water to the tube containing 5mg of Ferrozine. Mix by vortexing.
3. Prepare the 250 µM EDTA positive control in a separate tube by adding 5µL of the 50mM stock to 995µL of Assay Buffer.
4. Prepare test sample dilutions in Assay Buffer as necessary.
5. Make 6mL of Working $FeSO_4$ Solution by adding 60 µL of the 100x solution (from step 1) to 5.94mL of distilled water.
6. Add 50 µL of distilled water to 3 or 4 wells of the assay plate (background) and then add 50 µL of the Working $FeSO_4$ Solution to the remaining wells. (See Example Plate Set Up)
7. Add 50 µL of Assay Buffer to the background wells containing distilled water from step 6.
8. To prepare the maximal signal control, add 50 µL of Assay Buffer to 3 or 4 wells containing the Working $FeSO_4$ Solution (See Example Plate Set Up).
9. To prepare the assay inhibitor control, add 250µM EDTA to triplicate wells containing the Working $FeSO_4$ Solution (See Example Plate Set Up).
10. Add 50 µL of samples to triplicate wells containing the Working $FeSO_4$ Solution.
11. Make 12mL of Working Ferrozine Solution by adding 120 µL of the 100x Ferrozine solution (from step 2) to 11.88mL distilled water and mix.
12. To begin the assay, add 100 µL of the Working Ferrozine Solution to each well of the assay plate and incubate at room temperature for 10 minutes.
13. Determine absorbance using plate reader at a wavelength of 562 nm.

ALTERNATIVE ASSAY PROCEDURE

This includes an EDTA standard curve to determine EDTA equivalents of test samples.
Prepare a 500 µM EDTA solution by adding 10 µL 50mM EDTA to 990 µL Assay Buffer and mix.
Prepare the EDTA standards according to the following table:

Standard	Final Conc. (µM)	Assay Buffer (µL)	Volume (µL)	EDTA Solution
Std500	500	0	300	500 µM solution
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
Std15.63	15.63	150	150	Std31.25
Std7.86	7.81	150	150	Std15.63
Std0	0	150	0	0

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

Add 50 µL of each standard to duplicate wells on each assay plate used. (See Ex. Plate Set Up)

DATA ANALYSIS

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

1. Determine the background absorbance value by averaging the absorbance values of the background wells (no $FeSO_4$ or sample).
2. Subtract the background absorbance from all of the absorbance values.
3. Determine the maximal absorbance value by averaging the absorbance values of the wells containing $FeSO_4$, Ferrozine and Assay Buffer (from step 8, above).
4. Use the following equation to determine the ferrous ion chelating percent:

   Ferrous ion chelating (%) = 100 x $(Abs_{max} – Abs_{test})/Abs_{max}$

   Abs_{max} is determined in step 3.

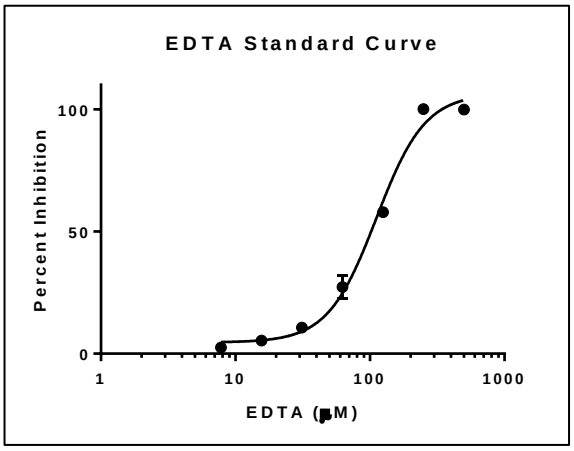
   Abs_{test} is the absorbance of the test sample minus background.

ALTERNATIVE DATA ANALYSIS

Values are determined as μM EDTA equivalents.

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

EDTA uM	Chelating Activity (%)			Avg.	Stdev
500	99.8	99.8	99.9	99.8	0.07
250	99.8	100.2	100.1	100.0	0.18
125	58.9	57.9	56.8	57.9	1.02
62.5	32.5	26.3	23.1	27.3	4.76
31.25	10.9	11.9	9.0	10.7	1.47
15.63	5.9	6.7	3.3	5.1	2.43
7.81	1.9	4.3	1.4	2.6	1.57



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.

EXAMPLE PLATE SET UP _____

	1			9			17			25	
	2			10			18			26	
	3			11			19			27	
	4			12			20			28	
	5			13			21			29	
	6			14			22			Max	Signal
	7			15			23		250µM	EDTA	
	8			16			24			Bkgd	

OR

500	EDTA		1			9			17		Bkgd
250			2			10			18		
125			3			11			19		
62.5			4			12			20		
31.25			5			13			21		Max
15.63			6			14			22		Signal
7.81			7			15			23		
0			8			16			24		

Background (Bkgd): 50 µL of distilled water + 50 µL of Assay Buffer + 100 µL Working Ferrozine Solution
Maximum Signal (Max Signal): 50 µL of $FeSO_4$ + 50 µL of Assay Buffer + 100 µL Working Ferrozine Solution

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

FERROUS IRON CHELATING ASSAY

Prior to assay, prepare working solutions of $FeSO_4$ and Ferrozine. Prepare EDTA positive control or alternatively, standard curve.

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Make necessary test compound dilutions in Assay Buffer.

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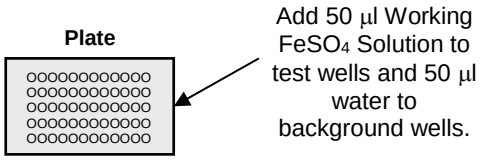
Add 50 µl/well Working $FeSO_4$ Solution to blank assay plate. Add 50 µl distilled water to triplicate wells for background.



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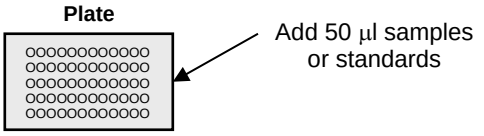
Add 50 µl/well samples or standards to assay plate.



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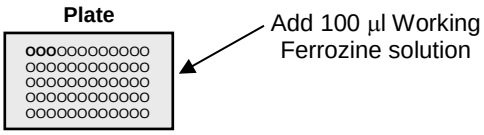
Start the assay by adding 100 µl/well Working Ferrozine Solution.



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Incubate at 25˚C (room temperature) for 10 minutes.

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Measure the optical density of each well at 562 nm using a spectrophotometer plate reader.

REFERENCES

1. Int. J. Food Sci. Nutr., **56**(7):491–499, 2005.
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 0.1 absorbance units different.

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