Cat. No. 300

Measure C-Fe®

Ascorbic Acid (High Sensitivity) Colorimetric Assay Kit (250 assays)

Quick User Guide

1. Storage conditions before opening: refrigerated at 4°C for half a year. (Ascorbate oxidase refrigerated at -80 °C)
2. Absorption spectrometer detection wavelength (nm): 595 nm
3. Standard or sample volume: 100 µL
4. 1X ascorbate oxidase solution addition amount: 10 µL (Add 10 µL oxidase-free ddH_2O to the control group).
5. The volume ratio of working solution：reaction solution (agent A), iron (III) chloride solution (agent B) and 1X Assay buffer solution (agent C) = 1:1:8
6. Reaction solution addition amount: 100 µL
7. Reaction time & temperature：15 mins at room temperature.
8. Working range：0~200 µM.

Introduction

Ascorbic acid is an important water-soluble antioxidant found in living organisms. The L-enantiomer is commonly known as Vitamin C (Figure 1). As a reducing agent, it provides electrons for various enzymatic and some non-enzymatic reactions required by all animals and plants. ◦ Its reducing ability can be an antioxidant to quickly scavenge reactive oxygen species (ROS). Ascorbic acid also plays an irreplaceable role in various physiological functions such as tissue growth, wound healing, synthesis of neurotransmitters, blood cholesterol levels, and elimination free radicals. However, the human body cannot synthesize vitamin C and must be ingested through diet. Scurvy results when vitamin C is deficient in the diet. In biological samples, various antioxidants, such as albumin, exist in the same area. Therefore, the detection method for quantitative analysis of ascorbic acid is easily affected by them, resulting in strong interference. Measure C-Fe® provides a fast, simple and sensitive method unaffected by other antioxidants to detect a variety of biological samples (eg: blood, urine, saliva, tears, tissue fluid, tissue/cell extracts and food ascorbic acid content in the extract).

Assay Principle

The detection theory of this set is based on the reduction ability provided by the electrons of antioxidants in the sample, which drives the reduction of Fe^{3+} into Fe^{2+} ions. Fe^{2+} ions react with the colorant in the set to produce a strong absorption spectrum change at 595 nm. This kit uses ascorbate oxidase (AO) to specifically destroy ascorbic acid and separates the electron reducing power provided by ascorbic acid in the sample from other antioxidants. Each test sample was assayed in duplicate, one with ascorbate oxidase treatment (+AO) and the other without ascorbate oxidase (-AO). Finally, the ascorbic acid concentration was deduced from the difference in OD595 readings (ΔAO) of the paired samples. This kit requires only a small amount of sample (100 µL) and the limit of detection (LOD) of ascorbic acid concentration can reach 0.016 µM. It provides a practical analysis tool for the change of ascorbic acid concentration in biological samples. This set can use a 96-well plate with a microplate reader or use a 0.5 mL cuvette

with the recommended spectrometer for measurement. Please refer to the operation steps for the operation method.

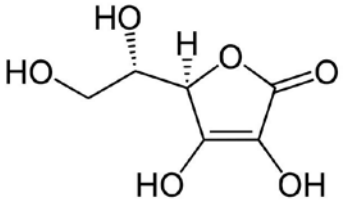

Figure 1. L-Ascorbic Acid Structure

Kit Components and Reagent Preparation

1. Color former (agent A): 3 mL brown glass bottle*1, used directly.
2. Iron (III) chloride solution (agent B): 3 mL translucent plastic tube*1, used directly.
3. 5X Assay buffer (agent C): 25 mL white plastic bottle*1, diluted to 1X with ddH_2O before use.
4. Ascorbic acid standard: 200 mg powder in brown pointed bottom plastic bottle*1, add 2 mL ddH_2O into 35 mg ascorbic acid powder to get 10X ascorbic acid standard solution (100 mM). The standards can be used immediately or divided into portions after reconstitution. Ascorbic acid solution should be stored at -20°C in 100 µL.
5. Ascorbate oxidase solution (50X): translucent plastic tube with green cap*1 and store at -80°C. Dilute to 1X with ddH_2O as needed before use (e.g. for 50 wells analysis, take 2 µL of 50X oxidase and dilute to 100 µL with ddH_2O. It can be used only once).

Package and Shipping information

Composition	Shipping Information	Store information before opening the package
1. Agent A: color former	Refrigerate at -20 °C.	Refrigerated at 4°C. Under proper storage conditions, it can be stored for half a year.
2. Agent B:Iron (III) chloride solution		
3. Agent C: 5X buffer solution		
4. Ascorbic Acid Standard		
5. Ascorbate oxidase (50X)		Refrigerated at -80 °C. Under proper storage conditions, it can be stored for half a year.

Note: Ascorbate oxidase must be frozen at -80°C.

Materials Not Supplied

1. 96-well microtiter plates
2. 10 kDa MWCO centrifugal filter (for high protein content samples)
3. Distillation-Distillation H_2O (ddH_2O)
4. Micropipettes
5. Conical tubes, microcentrifuge tubes, and bottles for sample and buffer preparation
6. Centrifuge and/or microfuge
7. Sonicator or tissue homogenizer
8. Microplate reader

Preparation of Samples

All samples should be analyzed immediately or stored for up to 2 months at -80°C after sampling. The test reading should be within the linear confidence interval of the standard. The high concentration of the sample may cause the interference by other components in the sample. Recommend to dilute with PBS as needed before testing. Always run a standard curve with samples.

Body fluids: Centrifuge tissue fluid at 12,000 rpm for 20 minutes at 4°C. After extracting the supernatant, samples should be tested immediately or frozen at -80°C.

Blood: Blood samples were collected with heparin tube* as anticoagulant. Centrifuge at 3,000 rpm for 10-15 minutes at 4°C. Carefully aspirate the upper yellow plasma supernatant. Samples should be tested immediately or frozen at -80°C.

Note: Hemolysis should be avoided. Limited use of heparin as an anticoagulant, EDTA will interfere with the color reaction.

Cell Lysates: Lyse 1-2 x 10^6 cells/mL by sonication or multiple freeze-thaw cycles in 4 volumes of cold 1X Assay Buffer. Centrifuge at 12,000 rpm for 15 minutes at 4ºC and remove insoluble cell material. A high concentration of protein may interfere with the assay. Filter the sample with a 10kDa MWCO centrifugal filter before assaying (to remove protein interference). Samples should be tested immediately or frozen at -80°C.

Tissue Lysates: 10 mg of tissue was homogenized/sonicated with 1~2 mL of 1X Assay Buffer (five-fold dilution of agent C) at 4°C. The supernatant was collected after centrifugation of the homogenate at 12,000 rpm for 15 min at 4°C. High concentrations of protein may interfere with the assay. 10 kDa protein centrifuge filter tubes can be used to exclude protein interference. Test the samples immediately or store it at -80°C.

Food Extracts: Wash uncooked food (e.g. fruits or vegetables) in water and homogenize 5g (fresh wet weight) in 100 mL distilled water for 30 seconds. Centrifuge the homogenate at 12,000 rpm for 15 min at 4°C and collect the supernatant. After extracting the supernatant, samples should be tested immediately or frozen at -80°C.

Assay Protocol and Preparation of Standard Curve

1. Thaw 10X ascorbic acid standard (100 mM) and dilute 10-fold with 1X Assay buffer. Serial dilutions of ascorbic acid standards (10 mM) were made to configure a standard curve. The configuration can refer to the following table:

Standard Tubes	Concentration (μM)	Source (μL)	1X Assay Buffer (μL)	Total volume (μL)
A	200	40 from ascorbic acid standard (10 mM)	1960	2000
B	100	750 from A tube	750	1500
C	50	750 from B tube	750	1500
D	25	750 from C tube	750	1500
E	20	100 from A tube	900	1000
F	10	100 from B tube	900	1000
G	2	100 from E tube	900	1000
H	1	100 from F tube	900	1000
I	0 (Blank)	--	1000	1000

2. Prepare the working solution: Mix the reaction solution(agent A), the iron (III) chloride solution (agent B) and 1X Assay buffer solution (diluted agent C) in a volume ratio of **1：1：8**.
   (For example: 100 mL of working solution = 10 mL of agent A + 10 mL of agent B

+ 80 mL 1X Assay buffer solution).

3. Add 100 µL of gradient concentration standard solution or sample to 96-well plate. (Note: Both samples and standards should be tested in pairs, one treated with ascorbate oxidase (+AO group) and the other without the enzyme (-AO group)).
4. Add 10 µL (1X ascorbate oxidase solution) to +AO group and 10 µL ddH_2O to -AO group, make sure the solution is well mixed and incubate the plate for 15 minutes at room temperature.
5. Add 100 µL of the working solution and incubate the plate for 15 minutes at room temperature. Keep out of light during the reaction.
6. Read absorbance on a microplate reader using 595 nm as the dominant wavelength. Make sure the instrument reading is within the linear range of the assay (0-2.5 OD), and dilute the sample if necessary.

Calculation of Results

1. Subtract the absorbance value of the experimental group containing ascorbate oxidase (A_{+AO}) from the value of the group without ascorbate oxidase (A_{-AO}) in [Standard/Blank]. The difference is caused by ascorbic acid. Absorbance (ΔAO), a standard curve was plotted against the mean value of the triplicate ΔAO (Figure 2). $(\Delta AO) = (A_{-AO}) - (A_{+AO})$
2. Compare the change in absorbance (ΔAO) of each sample to the standard curve to infer the amount of ascorbic acid in the sample.

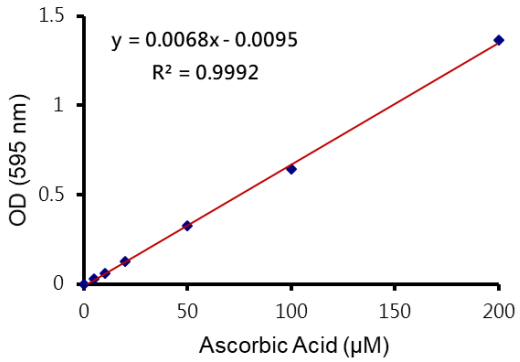

Figure 2. Ascorbic Acid Standard Curve

References

1. Tsao, Yu-Ting, et al., *Bioeng Transl Med.* **2020**,6(2), e10199.
2. Tsao, Yu-Ting, et al., *Antioxidants* **2022**, *11*, 397.

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