Ascorbate Oxidase Activity

Colorimetric Microplate Assay Kit

User Manual

Catalog # CAK1051

(Version 3.1F)

Detection and Quantification of Ascorbate Oxidase (AAO) Activity in Tissue extracts, Cell lysate Samples.

For research use only. Not for diagnostic or therapeutic procedures.

I. INTRODUCTION

Ascorbate oxidase (AAO) is an apoplastic enzyme involved in metabolism of plant ascorbate (AA). Ascorbate (AA) plays a key role in defense against oxidative stress and is particularly abundant in photosynthetic tissues. Over 90% of the ascorbate is localized in the cytoplasm, but a substantial proportion is exported to the apoplast.

Ascorbate Oxidase Activity Colorimetric Microplate Assay Kit provides a convenient tool for sensitive detection of ascorbate oxidase activity in a variety of samples. In this assay, ascorbic acid is oxidized by ascorbate oxidase. Then, the probe is reduced to ferrous ion ($Fe2^{+}$) by the residual ascorbic acid, which reacts with dye reagent to form a colored product. The color intensity reduction at 510nm is proportional to the ascorbate oxidase activity in the sample.

II. KIT COMPONENTS

Component	Volume	Storage
96-Well Microplate	1 plate	
Assay Buffer	30 ml x 4	4 °C
Substrate	Powder x 2	4 °C, keep in dark
Reaction Buffer A	20 ml x 1	4 °C
Probe	Powder x 1	4 °C
Reaction Buffer B	10 ml x 1	4 °C
Dye Reagent	Powder x 1	4 °C, keep in dark
Standard	Powder x 1	4 °C, keep in dark
Positive Control	Powder x 1	-20 °C
Technical Manual	1 Manual	

III. MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader to read absorbance at 510 nm
2. Distilled water
3. Pipettor, multi-channel pipettor
4. Pipette tips
5. Mortar
6. Centrifuge
7. Timer
8. Ice

IV. REAGENT PREPARATION

Standard: Briefly centrifuge prior to opening. Dissolve in 1 ml distilled water to generate 10 mmol/L of top standard solution. Then perform 2-fold serial dilutions of the top standard solution using distilled water to make the standard curve. The concentration of standard curve could be 10.0/5.0/2.5/1.25/0.625/0.312/0.156 mmol/L. Store at -20 °C for 1 month or 4°C for 3 days.

Substrate: Briefly centrifuge prior to opening. Dissolve each substrate vial in 0.25 ml Reaction Buffer A to generate substrate stock solution. Store at -20 °C for 1 week. Dilute to substrate working solution by adding 50 µl stock solution into 450 µl Reaction Buffer A. Prepare working solution fresh for immediate use.

Positive Control: Briefly centrifuge prior to opening. Dissolve in 0.1 ml Assay Buffer to generate stock solution. Dilute the stock solution 25-fold using Assay Buffer to prepare the Positive Control working solution (eg. 10 µl to 240 µl Assay Buffer). Store at -80 °C for 1 month.

Probe: Briefly centrifuge prior to opening. Dissolve in 1 ml distilled water to generate probe stock solution. Dilute the stock solution 5-fold using distilled water to prepare the probe working solution (eg. 200 µl to 800 µl distilled water). Store at -20 °C for 1 month or 4°C for 3 days.

Dye Reagent: Briefly centrifuge prior to opening. Dissolve in 1 ml distilled water before use. Keep in dark and store at -20 °C for 1 month or 4°C for 3 days.

Note: Divide into small aliquots to avoid repeated freeze-thaw cycles.

V. SAMPLE PREPARATION

1. For cell and bacteria samples

Collect cell or bacteria into centrifuge tube, discard the supernatant after centrifugation, add 1 ml Assay Buffer for 5×10^6 cell or bacteria (the quantity should be adjusted according to the actual situation of the sample), sonicate (with power 20%, sonicate 3s, interval 10s, repeat 30 times); centrifuged at 10000g 4 °C for 10 minutes, take the supernatant into a new centrifuge tube and keep it on ice for detection.

2. For tissue samples

Weigh out 0.1 g tissue (the quantity should be adjusted according to the actual situation of the sample), homogenize with 1 ml Assay Buffer on ice, centrifuged at 10000g 4 °C for 10 minutes, take the supernatant into a new centrifuge tube and keep it on ice for detection.

VI. ASSAY PROCEDURE

Add following reagents into the microplate:

Reagent*	Sample**	Control	Positive Control	Standard	Blank
Reaction Buffer A	80 µl	80 µl	80 µl	80 µl	80 µl
Distilled water	--	10 µl	--	20 µl	30 µl
Sample	10 µl	--	--	--	--
Positive control	--	--	10 µl	--	--
Substrate	10 µl	10 µl	10 µl	--	--
Mix and put at room temperature for 5 minutes					
Probe	10 µl	10 µl	10 µl	--	--
Standard	--	--	--	10 µl	--
Reaction Buffer B	80 µl	80 µl	80 µl	80 µl	80 µl
Dye Reagent	10 µl	10 µl	10 µl	10 µl	10 µl
Mix and put in the oven at 37 °C for 5 minutes, record absorbance measured at 510 nm					

Note:

*Reagents must be added sequentially and should not be premixed prior to addition.

**For unknown samples, we recommend doing a pilot experiment & testing several doses to ensure the readings are within the standard curve range. If the enzyme activity is lower, please add more samples into the reaction system; or increase the reaction time; if the enzyme activity is higher, please dilute the sample, or decrease the reaction time.

VIII. CALCULATION

Unit Definition: One unit of AAO is defined as the enzyme catalyzes the oxidation of 1 μmol of ascorbic acid per minute.

1. According to the slope of the standard curve

$$\text{Activity} = \frac{(OD_{Control} - OD_{Sample}) - \text{Intercept}}{\text{Slope} \times T} \times n \quad (U/mL)$$

2. According to one point of the standard OD value and concentration

2.1. According to the volume of sample

$$\text{Activity} = \frac{(C_{Standard} \times V_{Standard}) \times (OD_{Control} - OD_{Sample})}{(OD_{Standard} - OD_{Blank}) \times V_{Sample} \times T} \quad (U/mL)$$

2.2. According to the protein concentration of sample

$$\text{Activity} = \frac{(C_{Standard} \times V_{Standard}) \times (OD_{Control} - OD_{Sample})}{(OD_{Standard} - OD_{Blank}) \times (V_{Sample} \times C_{Protein}) \times T} \quad (U/mg/mL)$$

2.3. According to the weight of sample

$$\text{Activity} = \frac{(C_{Standard} \times V_{Standard}) \times (OD_{Control} - OD_{Sample})}{(OD_{Standard} - OD_{Blank}) \times (W \times V_{Sample} / V_{Assay}) \times T} \quad (U/g)$$

2.4. According to the quantity of cells or bacteria

$$\text{Activity} = \frac{(C_{Standard} \times V_{Standard}) \times (OD_{Control} - OD_{Sample})}{(OD_{Standard} - OD_{Blank}) \times (N \times V_{Sample} / V_{Assay}) \times T} \quad (U/10^4)$$

Slope: the absorbance slope of standard curve

n: the dilution factor

$C_{Protein}$: the protein concentration of sample, mg/mL

W: the weight of total sample, g

N: the quantity of total cell or bacteria sample, 10^4

$C_{Standard}$: the concentration of standard, μmol/mL

$V_{Standard}$: the volume of standard in assay procedure, mL

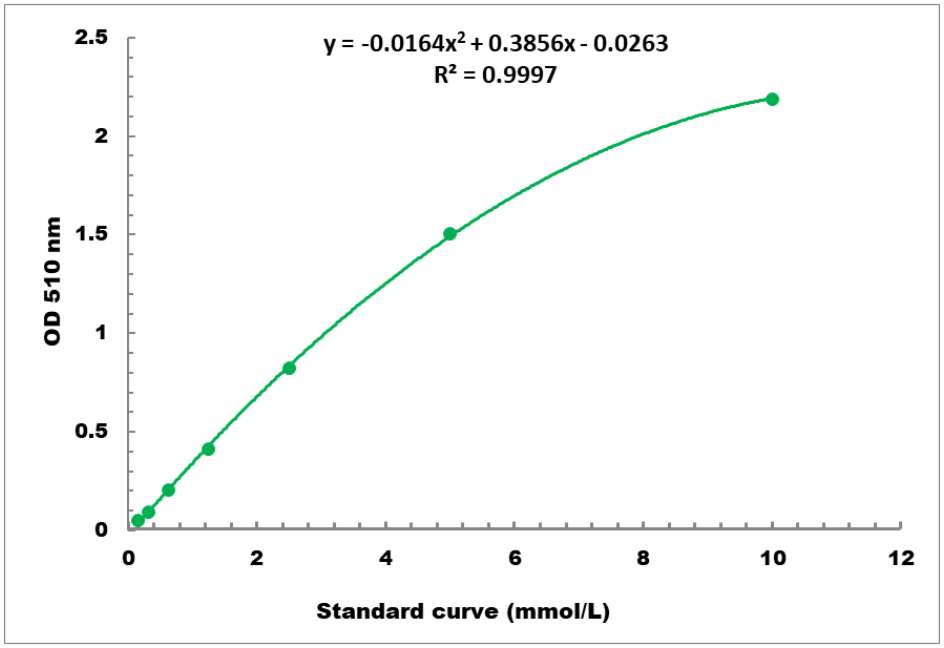
V_{Sample}: the volume of sample in assay procedure, mL

V_{Assay}: the volume of Assay Buffer in sample preparation, mL

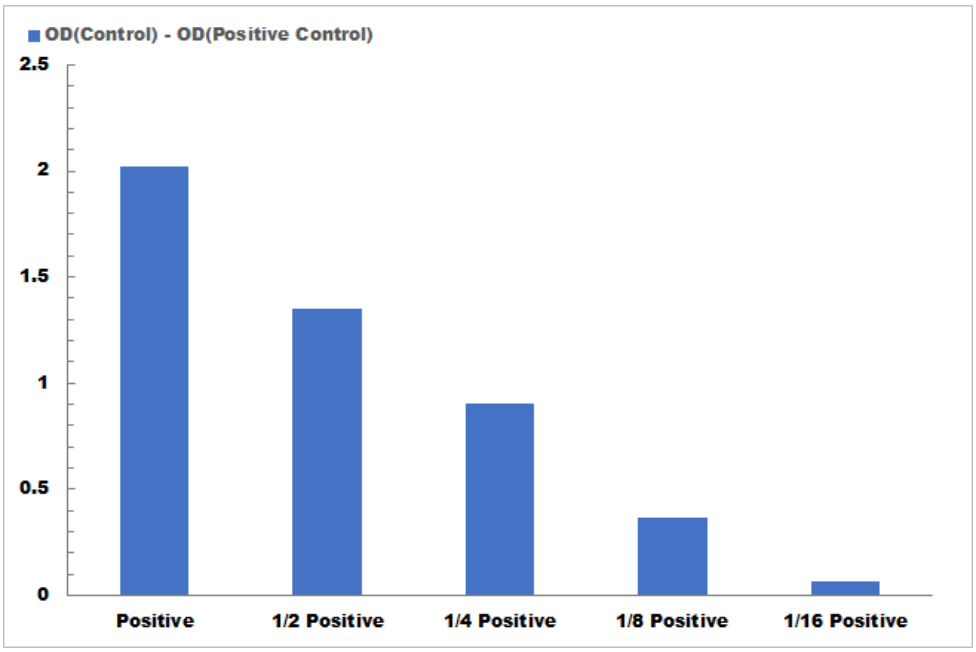
T: the reaction time, minute

VII. TYPICAL DATA

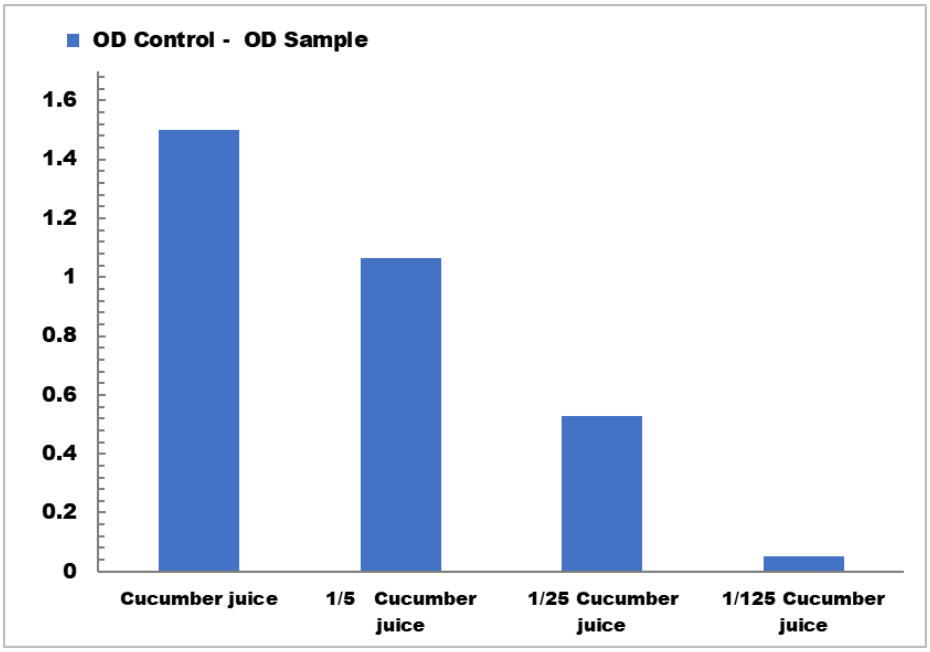
The standard curve is for demonstration only. A standard curve must be run with each assay.



Detection Range: 0.1 mmol/L - 10 mmol/L



Positive Control reaction in 96-well plate assay with decreasing the concentration



Determination of ascorbate oxidase in cucumber juices