Ascorbate Assay Kit

Item No. 700420

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700421	Ascorbate Standard	2 vials
700422	Ascorbate Assay Buffer	1 vial/25 ml
700423	Ascorbate DTPA	4 vials/500 µl
700424	Ascorbate Substrate	2 vials
700425	Ascorbate Developer	2 vials
400017	96-Well Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength between 340-350 nm and an emission wavelength between 420-430 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Methanol - A.C.S. Grade for chromatographic and UV spectrophotometric use

INTRODUCTION

Background

Ascorbate (L-Ascorbic acid or Vitamin C) is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates, and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is required for synthesis of the ascorbic acid immediate precursor 2-keto-1-gulonolactone. Therefore, humans must obtain ascorbate in their diet in order to survive. A lack of sufficient dietary ascorbate results in development of the fatal disease scurvy.

In humans, ascorbate acts as an electron donor for eight different enzymes, of which three participate in collagen hydroxylation by adding hydroxyl groups to proline or lysine in the collagen molecule increasing its stability.[1,2] Two other enzymes are necessary for synthesis of carnitine, which is essential to transport fatty acids into mitochondria for ATP generation.[3] Of the remaining three enzymes, one participates in the biosynthesis of norepinephrine from dopamine,[4] one adds amide groups to peptide hormones, greatly increasing their stability,[5] and one modulates tyrosine metabolism.[6]

Ascorbate also serves as an antioxidant and may be beneficial for reducing the risk of developing chronic diseases such as cancer, cardiovascular disease, and cataracts.[7] Ascorbate is also frequently used in the food industry as an antioxidant to prevent undesirable changes in color, taste, and odor.[8]

About This Assay

Cayman's Ascorbate Assay provides a reproducible and sensitive tool for assaying ascorbate from plasma, serum, urine, and fruit juices. The Ascorbate Assay utilizes the condensation reaction of dehydroascorbic acid (DHA) with *o*-Phenylenediamine (OPDA) to form a fluorescent product.[8] Ascorbate is first oxidized to DHA using 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL). DHA is then condensed with OPDA giving 3-(dihydroxyethyl)-furo[3,4-*b*]-quinoxaline-1-one (DFQ) which can be analyzed by fluorescence using an excitation wavelength between 340-350 nm and an emission wavelength between 420-430 nm.

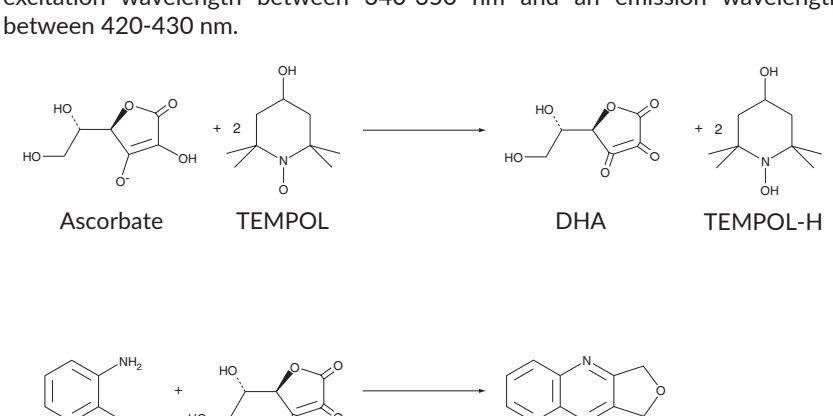


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Ascorbate Standard - (Item No. 700421)**

   Each vial contains a lyophilized powder of ascorbic acid that will be used to prepare the standard curve (see **Standard Preparation** on page 14).

2. **Ascorbate Assay Buffer - (Item No. 700422)**

   The vial contains 25 ml of 2 M Sodium Acetate, pH 5.5. Thaw the Assay Buffer at room temperature. This buffer should be used to prepare the Ascorbate Substrate (Item No. 700424) and Ascorbate Developer (Item No. 700425). When stored at 4°C, the thawed assay buffer is stable for six months.

3. **Ascorbate DTPA - (Item No. 700423)**

   Each vial contains 500 µl of a diethylenetriaminepentaacetic acid (DTPA) solution in sodium hydroxide. Thaw the vial contents at room temperature. The DTPA is ready to use as supplied. One vial contains enough reagent to prepare 20 ml of methanol/water solution (see Step 5 on page 8).

4. **Ascorbate Substrate - (Item No. 700424)**

   Each vial contains a lyophilized powder of 4-Hydroxy-TEMPOL. Reconstitute the contents of one vial with 6 ml of Ascorbate Assay Buffer (Item No. 700422). One vial contains enough substrate to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial. Any unused substrate solution can be stored at 4°C for up to one month.

5. **250 µM DTPA in Methanol/Water Solution**

   Determine the desired concentration of the methanol/water solution needed from the table below.

Used for evaluating:	Methanol (ml)	Water (ml)	DTPA (ml)	Final Methanol/H_2O/DTPA (v/v/v)
*Plasma/Serum	18	1.5	0.5	90:7.5:2.5
Urine	15	4.5	0.5	75:22.5:2.5
Fruit Juices	15	4.5	0.5	75:22.5:2.5

Table 1. Preparation of 250 µM DTPA in Methanol/Water

*Plasma and serum require 90% methanol to precipitate the proteins. After extraction, the final methanol concentration in the sample is approximately 75%. When preparing the ascorbate standards for evaluating plasma/serum, use 75:22.5:2.5 methanol/H_2O/DTPA. The methanol concentration in the standards needs to be the same as the final methanol concentration of the sample being evaluated.[8]

6. **Ascorbate Developer - (Item No. 700425)**

   Each vial contains a lyophilized powder of *o*-Phenylenediamine (OPD). Immediately prior to use, reconstitute the contents of one vial with 3 ml of Ascorbate Assay Buffer (Item No. 700422) and use within five minutes. Do not store reconstituted Ascorbate Developer. One vial contains enough developer to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial.

Sample Preparation

Plasma

Normal human plasma typically has an ascorbate concentration in the range of 60-80 µM.[9]

1. Collect blood using an anticoagulant such as heparin, EDTA, or sodium citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice. **Do not store the plasma at -80°C at this point, since ascorbate can degrade and requires stabilization with DTPA.**
3. Add 800 µl of (90:7.5:2.5) methanol/water/DTPA (see Table 1, page 8) to a micro-centrifuge tube. Add 200 µl of the collected plasma and vortex.
4. Incubate tube on ice for 10 minutes to precipitate the proteins.
5. After 10 minutes, centrifuge at 4°C and 12,000 x g for 10 minutes.
6. Collect the supernatant and store on ice. At this point, if not assaying the same day, freeze at -80°C. The plasma sample will be stable for one month while stored at -80°C.
7. Typically, plasma samples do not need to be diluted further before assaying.

Serum

Normal human serum typically has an ascorbate concentration in the range of 60-80 µM.

1. Collect blood without using an anticoagulant.
2. Allow the blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. **Do not store the serum at -80°C at this point, since ascorbate can degrade and requires stabilization with DTPA.**
4. Add 800 µl of (90:7.5:2.5) methanol/water/DTPA (see Table 1, page 8) to a micro-centrifuge tube. Add 200 µl of the collected serum and vortex.
5. Incubate tube on ice for 10 minutes to precipitate the proteins.
6. After 10 minutes, centrifuge at 4°C and 12,000 x g for 10 minutes.
7. Collect the supernatant and store on ice. At this point, if not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.
8. Typically, serum samples do not need to be diluted further before assaying.

Urine

Normal human urine typically has an ascorbate concentration in the range of 95-270 µM.[10]

1. Collection of urine does not require any special treatment.
2. Typically urine samples require dilutions of at least 1:10 or greater. Dilute the urine using (75:22.5:2.5) methanol/water/DTPA (see Table 1, page 8) in a micro-centrifuge tube and vortex.
3. Incubate the tube on ice for 10 minutes to precipitate any proteins.
4. After 10 minutes, centrifuge at 4°C and 12,000 x g for 10 minutes.
5. Collect the supernatant and store on ice. At this point, if not assaying the same day, freeze at -80°C. The urine sample will be stable for one month while stored at -80°C.

It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

Fruit Juices

1. Add juice sample to a micro-centrifuge tube and centrifuge for one minute at 16,000 x g.
2. Transfer the supernatant to a new centrifuge tube.
3. Dilute the supernatant in the range of 1:10 to 1:250 using (75:22.5:2.5) methanol/water/DTPA (see Table 1, page 8). *NOTE: Several dilutions should be made which will allow the ascorbate amount to fall within the range of the assay.*
4. Incubate the tube on ice for 10 minutes.
5. After 10 minutes, centrifuge at 4°C and 12,000 x g for 10 minutes.
6. Collect the supernatant and store on ice. At this point, if not assaying the same day, freeze at -80°C. The juice sample will be stable for one month while stored at -80°C.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that each sample and standard be assayed at least in duplicate (triplicate is preferred) along with duplicate or triplicate wells designated as background wells for each sample. A typical layout of samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
B	B	B	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
C	C	C	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
D	D	D	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
E	E	E	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19
F	F	F	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
G	G	G	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20
H	H	H	S4	S4	S8	S8	S12	S12	S16	S16	S20	S20

A-H = Standards
B1-B20 = Background Sample Wells
S1-S20 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 250 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- The assay is performed at 25°C.
- Monitor the fluorescence with an excitation wavelength between 340-350 nm and an emission wavelength between 420-430 nm.

Standard Preparation

Reconstitute a vial of the Ascorbate Standard (Item No. 700421) with 1 ml of water to obtain a stock solution of 1 mM. Take eight clean test tubes and label them A-H. Add the amount of 1 mM ascorbate and the prepared (75:22.5:2.5) methanol/water/DTPA solution to each tube as described in Table 2. We recommend that you store these diluted standards in an ice bath for no more than one hour.

Tube	1 mM Ascorbate (µl)	MeOH/H_2O/DTPA (µl)	Ascorbate Concentration (µM)
A	0	1,000	0
B	5	995	5
C	10	990	10
D	25	975	25
E	50	950	50
F	75	925	75
G	100	900	100
H	150	850	150

Table 2. Preparation of Ascorbate Standard Curve

Performing the Assay

NOTE: Use the (75:22.5:2.5) methanol/water/DTPA solution for all standard, sample, and sample background wells.

1. **Blank Wells -** Add 100 µl of standard tube A per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 12).
2. **Standard Wells -** Add 50 µl of methanol/water/DTPA solution and 50 µl of standard (tubes B-H) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 12).
3. **Sample Background Wells -** Add 50 µl of methanol/water/DTPA solution and 50 µl of sample to at least three wells. Add 100 µl of Ascorbate Assay Buffer (Item No. 700422).
4. **Sample Wells -** Add 50 µl of methanol/water/DTPA solution and 50 µl of the sample to at least three wells. To obtain reproducible results, the amount of ascorbate added to the wells should fall within the range of the assay. When necessary, samples should be diluted with methanol/water/DTPA solution.
5. Add 100 µl of the reconstituted Ascorbate Substrate to all blank, standard and sample wells. DO NOT add the reconstituted Ascorbate Substrate to the sample background wells.
6. Cover the plate with the plate cover, and incubate for 10 minutes at 25°C.
7. Prepare the Ascorbate Developer within five minutes prior to adding to the wells. Add 50 µl of the Developer to all wells being used, including the blank, standards, sample, and sample background. Cover, and incubate for five minutes at 25°C while in the dark.
8. Remove the plate cover and read using an excitation wavelength between 340-350 nm and an emission wavelength between 420-430 nm.

Well	Standard (µl)	Methanol/ Water/DTPA (µl)	Sample (µl)	Buffer (µl)	Ascorbate Substrate (µl)
Blank	100 (tube A)				100
Standard	50	50			100
Sample Background		50	50	100	
Sample		50	50		100

Table 3. Pipetting summary

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard, sample, and sample background.
2. Subtract the fluorescence value of the sample background from the fluorescence of the sample wells to yield the corrected sample fluorescence (CSF).
3. Determine the average fluorescence of the standards. Subtract the fluorescence value of standard A (0 µM) from itself and all other standards. This is the corrected fluorescence.
4. Plot the corrected fluorescence values (from step 3 above) of each standard as a function of the final concentration of ascorbate from Table 2. See Figure 3, on page 19, for a typical standard curve.
5. Calculate the ascorbate concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the corrected sample fluorescence (CSF) for each sample.

$$\text{Ascorbate Concentration (µM)} = \left[\frac{\text{CSF - (y-intercept)}}{\text{Slope}}\right] \text{x Sample dilution}$$

Ascorbate Concentration (µg/L) = (µM)(176.12)
NOTE: Ascorbate molecular weight = 176.12

Performance Characteristics

Precision:

When a series of 48 plasma and urine measurements were performed on the same day, the intra-assay coefficient of variation was 4.5% and 3.2%, respectively. When a series of 16 plasma and urine measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.6% and 3.1%, respectively.

Assay Range:

Under the standardized conditions for the assay described in this booklet, the dynamic range of the kit is 0-150 µM ascorbate.

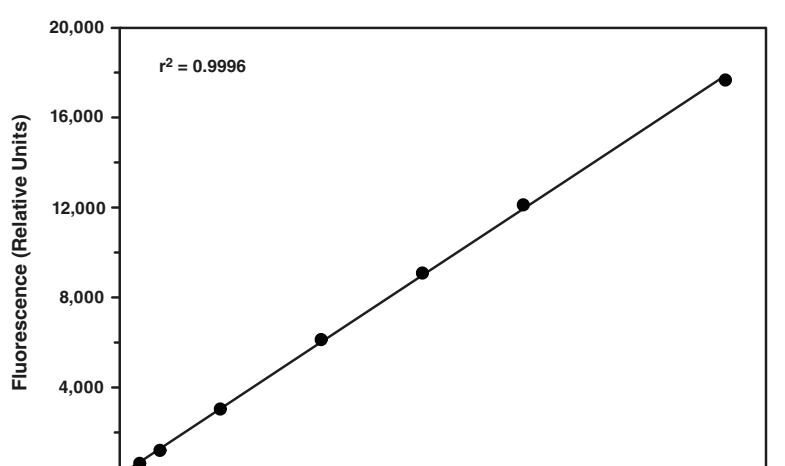


Figure 3. Ascorbate standard curve

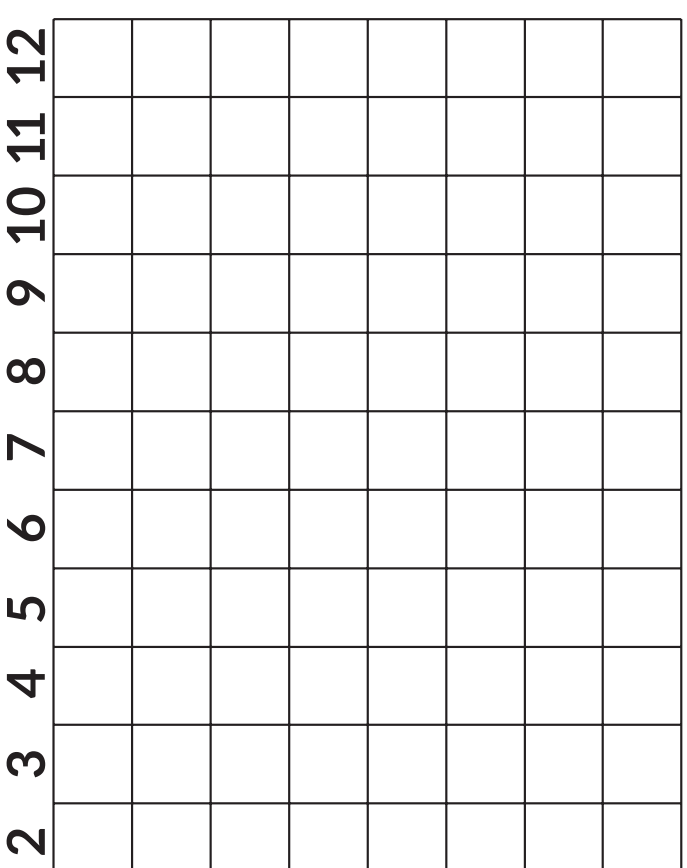
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No Ascorbate concentration was obtained above background in the sample	A. The ascorbate concentration is too low to detect B. The sample does not contain ascorbate	A. Re-assay the sample using a lower dilution
Ascorbate concentration was above the highest point in the standard curve	A. The ascorbate concentration was too high in the sample B. The sample was too concentrated	Dilute samples with the 75:22.5:2.5 in methanol/ water/DTPA solution (see Table 1, page 8) and re-assay; NOTE: Remember to account for the dilution factor when calculating ascorbate concentration
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read

References

1. Gropper, S.S., Smith, J.L., and Groff, J.L. The water-soluble vitamins, Chapter 9, *in* Advanced Nutrition and Human Metabolism. 4, Thomson Wadsworth, 259-275 (2005).
2. Peterkofsky, B. Ascorbate requirement for hydroxylation and secretion of procollagen: Relationship to inhibition of collagen synthesis in scurvy. *Am. J. Clin. Nutr.* **54(6)**, 1135S-1140S (1991).
3. Rebouche, C.J. Ascorbic acid and carnitine biosynthesis. *Am. J. Clin. Nutr.* **54(6)**, 1147S-1152S (1991).
4. Diliberto, E.J., Jr., Daniels, A.J., and Viveros, O.H. Multicompartmental secretion of ascorbate and its dual role in dopamine β-hydroxylation. *Am. J. Clin. Nutr.* **54(6)**, 1163S-1172S (1991).
5. Englard, S. and Seifter, S. The biochemical functions of ascorbic acid. *Annu. Rev. Nutr.* **6**, 365-406 (1986).
6. Eipper, B.A., Milgram, S.L., Husten, E.J., *et al.* Peptidylglycine α-amidating monooxygenase: A multifunctional protein with catalytic, processing, and routing domains. *Protein Sci.* **2**, 489-497 (1993).
7. Padayatty, S.J., Katz, A., Wang, Y., *et al.* Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* **22(1)**, 18-35 (2003).
8. Vislisel, J.M., Schafer, F.Q., and Buettner, G.R. A simple and sensitive assay for ascorbate using a plate reader. *Anal. Biochem.* **365(1)**, 31-39 (2007).
9. Schorah, C.J., Downing, C., Piripitsi, A., *et al.* Total vitamin C, ascorbic acid, and dehydroascorbic acid concentrations in plasma of critically ill patients. *Am. J. Clin. Nutr.* **63(5)**, 760-765 (1996).
10. Koshiishi, I. and Imanari, T. Measurement of ascorbate and dehydroascorbate contents in biological fluids. *Anal. Chem.* **69(2)**, 216-220 (1997).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.