

## **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) 975-3999. We cannot accept any returns without prior authorization.



**WARNING:** This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

## Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

## If You Have Problems

### Technical Service Contact Information

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

**Fax:** 734-971-3641

**Email:** techserv@caymanchem.com

**Hours:** M-F 8:00 AM to 5:30 PM EST

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 repeat 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

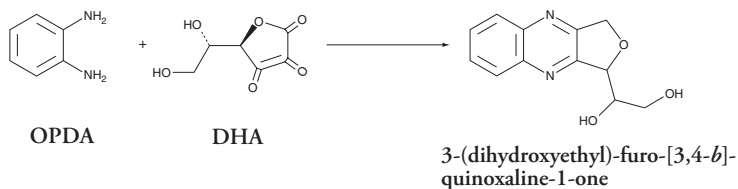
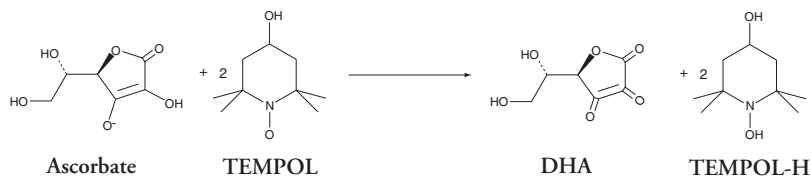
Ascorbate (L-Ascorbic acid or Vitamin C) is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates, and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is required for synthesis of the ascorbic 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.<sup>1,2</sup> Two other enzymes are necessary for synthesis of carnitine, which is essential to transport fatty acids into mitochondria for ATP generation.<sup>3</sup> Of the remaining three enzymes, one participates in the biosynthesis of norepinephrine from dopamine,<sup>4</sup> one adds amide groups to peptide hormones, greatly increasing their stability,<sup>5</sup> and one modulates tyrosine metabolism.<sup>6</sup>

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.<sup>7</sup> Ascorbate is also frequently used in the food industry as an antioxidant to prevent undesirable changes in color, taste, and odor.<sup>8</sup>

### 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.<sup>8</sup> 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 $\mu$ M 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 <sub>2</sub> O/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  $\mu$ M 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<sub>2</sub>O/DTPA. The methanol concentration in the standards needs to be the same as the final methanol concentration of the sample being evaluated.<sup>8</sup>

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

Each vial contains a lyophilized powder of *o*-Phenylenediamine (OPDA). Reconstitute the contents of one vial with 3 ml of Ascorbate Assay Buffer (Item No. 700422). One vial contains enough developer to assay 60 wells. If additional wells are being utilized, then reconstitute the second vial. Any unused solution can be stored at 4°C for 24 hours in the dark.

## Sample Preparation

### Plasma

Normal human plasma typically has an ascorbate concentration in the range of 60-80  $\mu$ M.<sup>9</sup>

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  $\mu$ l of (90:7.5:2.5) methanol/water/DTPA (see Table 1, page 8) to a micro-centrifuge tube. Add 200  $\mu$ l 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  $\mu\text{M}$ .

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  $\mu\text{l}$  of (90:7.5:2.5) methanol/water/DTPA (see Table 1, page 8) to a micro-centrifuge tube. Add 200  $\mu\text{l}$  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  $\mu\text{M}$ .<sup>10</sup>

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.

*NOTE: Ascorbate values from urine samples can be standardized using Cayman's Creatinine Assay Kit (Item No. 500701).*

## Fruit Juices

1. Add juice sample to a micro-centrifuge tube and centrifuge for 1 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.

## 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 23).

|   | 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  $\mu$ l 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 <sub>2</sub> O/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/<br>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 18, 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.

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

$$\text{Ascorbate Concentration } (\mu\text{g/L}) = (\mu\text{M})(176.12)$$

*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  $\mu\text{M}$  ascorbate.

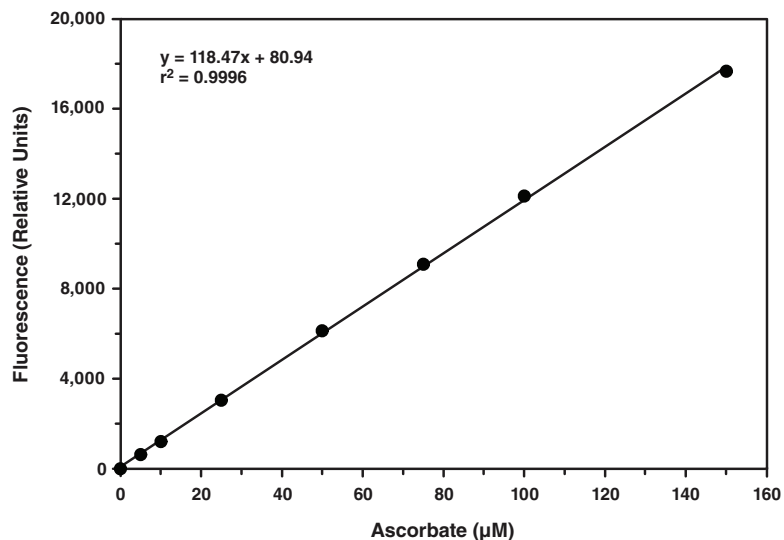


Figure 3. Ascorbate standard curve

## RESOURCES

### Troubleshooting

| Problem   | Possible Causes   | Recommended Solutions  |
|---|---|--|
| Erratic values; dispersion of duplicates/triplicates                      | A. Poor pipetting/technique<br>B. Bubble in the well(s)   | A. Be careful not to splash the contents of the wells<br>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<br>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<br>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

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10. Koshiishi, I. and Imanari, T. Measurement of ascorbate and dehydroascorbate contents in biological fluids. *Anal. Chem.* **69(2)**, 216-220 (1997).

## Related Products

Aconitase Assay Kit - Item No. 705502  
Antioxidant Assay Kit - Item No. 709001  
Catalase Assay Kit - Item No. 707002  
 $\gamma$ -CEHC EIA Kit (plasma and serum) - Item No. 10010621  
Glutathione Assay Kit - Item No. 703002  
Glutathione Peroxidase Assay Kit - Item No. 703102  
Glutathione Reductase Assay Kit - Item No. 703202  
Glutathione S-Transferase Assay Kit - Item No. 703302  
8-hydroxy-2-deoxy-Guanosine EIA Kit - Item No. 589320  
HIF-1 $\alpha$  Transcription Factor Assay Kit - Item No. 10006910  
Hydrogen Peroxide (urinary) Assay Kit - Item No. 706011  
8-Isoprostane EIA Kit - Item No. 516351  
oxLDL- $\beta$ 2GPI (human) ELISA Kit - Item No. 10007893  
Lipid Hydroperoxide (LPO) Assay Kit - Item No. 705002  
S-Nitrosylated Protein Detection Kit - Item No. 10006518  
*ent*-Prostaglandin F<sub>2 $\alpha$</sub>  EIA Kit - Item No. 10010382  
Protein Carbonyl Assay Kit - Item No. 10005020  
Superoxide Dismutase Assay Kit - Item No. 706002  
TBARS Assay Kit - Item No. 10009055  
Thioredoxin Reductase Assay Kit - Item No. 10007892  
Thiol Detection Assay Kit - Item No. 700340  
Xanthine Oxidase Assay Kit - Item No. 10010895

## Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.

|    |   |   |   |   |   |   |   |   |
|----|---|---|---|---|---|---|---|---|
| 12 |   |   |   |   |   |   |   |   |
| 11 |   |   |   |   |   |   |   |   |
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## NOTES

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