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# Product Information

**Ascorbic Acid Assay Kit II**

Catalog Number **MAK075**
Storage Temperature –20 °C

## TECHNICAL BULLETIN

### Product Description

Ascorbic Acid, also known as Vitamin C, is a six-carbon lactone produced by plants and some animal species but not by humans and other primates. Ascorbic acid functions as an enzymatic cofactor for multiple enzymes, serving as an electron donor for monooxygenases and dioxygenases. Ascorbic acid also functions as a powerful antioxidant, particularly in regards to reactive oxygen species.

In this assay, ascorbic acid concentration is determined using the Ferric Reducing/Antioxidant and Ascorbic Acid (FRASC) assay. In this assay, $Fe^{3+}$ is reduced to $Fe^{2+}$ by antioxidants present in the sample, which results in a colorimetric (593 nm) product. The addition of ascorbate oxidase to parallel samples oxidizes any ascorbic acid present allowing for the measurement of the ascorbic acid concentration.

This kit is suitable for use with cell and tissue culture supernatants as well as serum, plasma, urine, and other biological fluids.

### Components

The kit is sufficient for 100 assays in 96 well plates.

| | |
|---|---|
| FRASC Buffer<br>Catalog Number MAK075A | 25 mL |
| Ascorbic Acid Probe<br>Catalog Number MAK075B | 1 mL |
| Iron Chloride ($FeCl_3$) Solution<br>Catalog Number MAK075C | 1 mL |
| Ascorbate Oxidase<br>Catalog Number MAK075D | 1 vl |
| Ascorbic Acid Standard, 20 µmole<br>Catalog Number MAK075E | 1 vl |

### Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader
- 10 kDa Molecular Weight Cut-Off (MWCO) Spin Filter

### Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

### Preparation Instructions

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

FRASC Buffer – Allow buffer to come to room temperature before use.

Ascorbic Acid Probe – Ready-to-use as supplied. Stable for two months at room temperature.

Iron Chloride Solution – Ready-to-use as supplied. Stable for two months at room temperature.

Ascorbate Oxidase – Reconstitute with 500 µL of water. Mix well by pipetting, then aliquot and store, at –20 °C. Use within 2 months of reconstitution and keep cold while in use.

Ascorbic Acid Standard – Reconstitute in 200 µL of water to generate a 100 mM (100 nmole/µL) Ascorbic Acid Standard solution. Mix well by pipetting, then aliquot and store at –20 °C. Use within 2 months of reconstitution and keep cold while in use.

### Storage/Stability

The kit is shipped on wet ice. Storage at –20 °C, protected from light, is recommended.

**Procedure**
All samples and standards should be run in duplicate.

Ascorbic Acid Standards for Colorimetric Detection
Dilute 10 µL of the 100 mM (100 nmole/µL) Ascorbic Acid Standard Solution with 990 µL of water to prepare a 1 mM (1 nmole/µL) standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 1 mM ascorbic acid standard solution into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add water to each well to bring the volume to 100 µL.
Note: Diluted ascorbic acid standard is not stable and should be prepared fresh for each time.

Sample Preparation
Tissue (10 mg) or cells ($2 \times 10^6$) should be rapidly homogenized in 4 volumes of cold FRASC buffer. Centrifuge at 13,000 × *g* for 10 minutes at 4 °C to remove insoluble material. High concentrations of proteins may interfere with the assay. If this is an issue, protein may be removed with a 10 kDa MWCO spin filter. The soluble fraction may be assayed directly.

Bring samples to a final volume of 100 µL with water.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

This assay measures total antioxidant capacity in a cell. To measure ascorbic acid content, it is necessary to set up a blank for each sample. Ascorbate oxidase added to the blank sample will oxidize the ascorbic acid.

Assay Reaction
1. Add 10 µL of water to the sample and standard wells and 10 µL of Ascorbate Oxidase to the sample blank wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 15 minutes.

2. Set up the Master Reaction Mix according to the scheme in Table 1. 100 µL of the Master Reaction Mix is required for each reaction (well).

**Table 1.**
Master Reaction Mix

| **Reagent** | **Volume** |
|---|---|
| FRASC Buffer | 80 µL |
| Ascorbic Acid Probe | 10 µL |
| Iron Chloride Solution | 10 µL |

3. Add 100 µL of the Master Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 2–3 minutes at room temperature. In this assay, ascorbic acid reacts quickly with the probe, while other antioxidants react more slowly with the probe. The longer the incubation time, the higher the background will be.

4. Measure the absorbance at 593 nm ($A_{593}$) for maximum sensitivity. Wavelengths between 545–600 nm may be used as the probe will give 90% of maximum absorbance in this range.

**Results**

Calculations

The background for the assays is the value obtained for the 0 (blank) Ascorbic Acid Standard. Correct for the background by subtracting the 0 (blank) value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate Ascorbic Acid standards to plot a standard curve.

Note: A new standard curve must be set up each time the assay is run.

Subtract the blank sample value (containing Ascorbate Oxidase) from the sample reading to obtain the corrected measurement. Using the corrected measurement, the amount of Ascorbic Acid present in the sample may be determined from the standard curve.

Concentration of Ascorbic Acid

$$S_a/S_v = C$$

$S_a$ = Amount of Ascorbic Acid in unknown sample (nmole) from standard curve
$S_v$ = Sample volume (μL) added into the wells
C = Concentration of Ascorbic Acid in sample

Ascorbic Acid molecular weight: 176.12 g/mole

Sample Calculation

Amount of Ascorbic Acid ($S_a$) = 5.84 nmole (from standard curve)
Sample volume ($S_v$) = 100 μL

Concentration of Ascorbic Acid in sample

$$5.84 \text{ nmole}/100 \text{ μL} = 0.058 \text{ nmole}/\text{μL}$$

$$0.058 \text{ nmole}/\text{μL} \times 176.12 \text{ ng/nmole} = 10.29 \text{ ng}/\text{μL}$$

**Troubleshooting Guide**

| Problem | Possible Cause | Suggested Solution |
|---|---|---|
| Assay not working | Ice Cold Assay Buffer | Assay Buffer must be at room temperature |
| | Omission of step in procedure | Refer and follow Technical Bulletin precisely |
| | Plate reader at incorrect wavelength | Check filter settings of instrument |
| | Type of 96 well plate used | For colorimetric assays, use clear plates |
| Samples with erratic readings | Samples prepared in different buffer | Use the Assay Buffer provided or refer to Technical Bulletin for instructions |
| | Cell/Tissue culture samples were incompletely homogenized | Repeat the sample homogenization, increasing the length and extent of homogenization step. |
| | Samples used after multiple freeze-thaw cycles | Aliquot and freeze samples if samples will be used multiple times |
| | Presence of interfering substance in the sample | If possible, dilute sample further |
| | Use of old or inappropriately stored samples | Use fresh samples and store correctly until use |
| Lower/higher readings in samples and standards | Improperly thawed components | Thaw all components completely and mix gently before use |
| | Use of expired kit or improperly stored reagents | Check the expiration date and store the components appropriately |
| | Allowing the reagents to sit for extended times on ice | Prepare fresh Master Reaction Mix before each use |
| | Incorrect incubation times or temperatures | Refer to Technical Bulletin and verify correct incubation times and temperatures |
| | Incorrect volumes used | Use calibrated pipettes and aliquot correctly |
| Non-linear standard curve | Use of partially thawed components | Thaw and resuspend all components before preparing the reaction mix |
| | Pipetting errors in preparation of standards | Avoid pipetting small volumes |
| | Pipetting errors in the Reaction Mix | Prepare a Master Reaction Mix whenever possible |
| | Air bubbles formed in well | Pipette gently against the wall of the tubes |
| | Standard stock is at incorrect concentration | Refer to the standard dilution instructions in the Technical Bulletin |
| | Calculation errors | Recheck calculations after referring to Technical Bulletin |
| | Substituting reagents from older kits/lots | Use fresh components from the same kit |
| Unanticipated results | Samples measured at incorrect wavelength | Check the equipment and filter settings |
| | Samples contain interfering substances | If possible, dilute sample further |
| | Sample readings above/below the linear range | Concentrate or dilute samples so readings are in the linear range |

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