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ab219928 Ascorbic Acid Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of Ascorbic Acid in cell lysates.

This product is for research use only and is not intended for diagnostic use.

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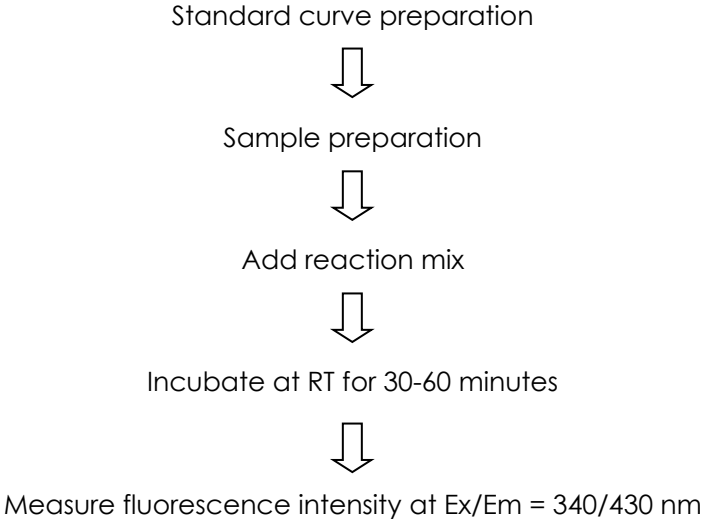
1. Overview

Ascorbic Acid Assay Kit (Fluorometric) (ab219928) provides a sensitive fluorescent assay for quantifying total ascorbic acid in cell lysate. This assay is based on the enzymatic oxidation of ascorbic acid to dehydroascorbate acid (DHA). DHA can then in turn be detected by our Blue Dye with a fluorescence microplate reader at Ex/Em = 340/430 nm. The assay can detect 1µM of total ascorbate.

This product can also be used to quantify the ratio of DHA to ascorbic acid. Under physiological conditions, DHA concentrations are extremely low. However, extracellular ascorbic acid is massively oxidized to DHA in certain neuropathological diseases, leading to an increase in the uptake of DHA in neurons, which can in turn result in neuronal damage.

L-Ascorbic Acid (also called Vitamin C) is a critical metabolite for both plant and animals in cell division, growth and defense. Ascorbate is produced from glucose in the liver of most mammalian species. For humans ascorbate has to be obtained from food to survive, and a lack of sufficient Vitamin C can result in scurvy, and may eventually lead to death. As an antioxidant ascorbate can reduce the risk of developing chronic disease such as cancer and cardiovascular disease. In food industry, ascorbic acid and its sodium, potassium, and calcium salts are commonly used as antioxidant food additives to prevent undesired color and taste.

2. Protocol Summary



3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Assay Buffer	20 mL	-20°C	-20°C
Ascorbic Acid Standard	3.52 mg	-20°C	-20°C
Blue Dye	1 vial	-20°C	-20°C
Enzyme Mix (lyophilized)	2 vials	-20°C	-20°C
DMSO	100 µL	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence at Ex/Em = 340/430 nm
- Double distilled water (ddH_2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom, preferably black
- Dounce homogenizer (if using tissue)
- (Optional) Mammalian Cell Lysis Buffer 5X (ab179835)

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 Ascorbic Acid Standard (3.52 mg):

Reconstitute the Ascorbic Acid Standard in 200 µL of dd H_2O to generate a 100 mM Ascorbic Acid Standard Stock Solution. Keep on ice while in use. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze-thaw cycles.

9.3 DMSO:

Ready to use as supplied. Warm by placing in a 37°C bath for 1-5 min to thaw the DMSO solution before use.

Δ Note: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for a few minutes at 37°C. Repeat this step every time probe is needed.

Store at -20°C. Avoid repeated freeze-thaw cycles.

9.4 Blue Dye (light-sensitive):

Prepare a **200X Blue Dye** stock solution by adding 50 µL of DMSO to the vial of Blue Dye. Mix well by pipetting up and down. Aliquot 200X Blue Dye stock solution so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Avoid repeated freeze-thaw cycles.

9.5 Enzyme Mix:

Prepare a **50X Enzyme Mix** stock solution by adding 100 µL of ddH_2O to one vial of Enzyme Mix and mix well.

Store unused **50X Enzyme Mix** stock solution at -20°C, avoid repeated freeze-thaw cycles.

Δ Note: 100 µL of 50X Enzyme mix is enough for 1 x 96 plate.

Store undiluted Enzyme Mix at -20°C. Avoid repeated freeze-thaw cycles.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 1 mM Ascorbic Acid (AA) standard solution by diluting 10 µL of the 100 mM AA Stock solution (Step 9.2) into 990 µL of ddH_2O. Mix well by pipetting up and down.

10.2 Using the 1 mM Ascorbic Acid standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Sample to dilute	Volume standard in well (µL)	Assay Buffer (µL)	End conc Ascorbic Acid in well
1	1 mM	450	0	1000 µM
2	Std #1	300	600	300 µM
3	Std #2	300	600	100 µM
4	Std #3	300	600	30 µM
5	Std #4	300	600	10 µM
6	Std #5	300	600	3 µM
7	Std #6	300	600	1 µM
8 (blank)	0	0	200	0 µM

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell lysates:

Δ Note: For ease of use, mammalian adherent or suspension cells lysates can be easily prepared using Mammalian Cell Lysis Buffer 5X (ab179835). Follow product protocol and proceed to Section 12.

11.1.1 Harvest the number of cells necessary for each assay (initial recommendation: 2-5 x 10^5 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend or scrape cells in 100 µL of cold PBS.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Centrifuge 5 minutes at 4°C at 13,000 x*g* in a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Keep on ice.

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure – AA only

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- The protocol describe in this section is for 1 x 96-well plate. To perform the assay in a 384-wp, scale down volumes by half.

12.1 Reaction wells set up:

- Blank control = 50 µL Assay Buffer.
- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with Assay Buffer).

12.2 Run Ascorbic Acid assay:

12.2.1 Prepare a Total Ascorbic Assay (AA) Reaction Mix as described in the table below. The volume given in the table is enough for 1 x 96-well plate.

Component	Total AA Reaction Mix
Assay Buffer	4.9 mL
50X Enzyme Mix	100 µL
Blue Dye	25 µL
Total volume	5.025 mL

Δ Note: if not running a full plate, prepare the reaction mix proportionally to Total AA assay (ie, for 1mL total AA assay, use 20 µL 50X Enzyme Mix + 5 µL 200X Blue Dye + 975 µL Assay Buffer).

12.2.2 Add 50 µL of Total AA Reaction Mix into each reaction well (total volume: 100 µL/well).

12.2.3 Incubate reaction mixture at room temperature for 30-60 minutes, protected from light.

12.3 Measurement:

12.3.1 Measure Fluorescence increase with a fluorescence plate reader at Ex/Em = 340/430 nm (cut off: 420 nm).

13. Assay Procedure – DHA/AA ratio

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- The protocol describe in this section is for 1 x 96-well plate. To perform the assay in a 384-wp, scale down volumes by half.

13.1 Reaction wells set up:

Δ Note: set up one plate to measure total AA and one plat to measure total DHA.

- Blank control = 50 µL Assay Buffer.
- Standard wells = 50 µL standard dilutions.
- Sample wells = 1-50 µL samples (adjust volume to 50 µL/well with Assay Buffer).

13.2 Run DHA/AA assay:

13.2.1 Prepare Total Ascorbic Assay (AA) Reaction Mix and DHA Reaction Mix as described in the table below. The volume given in the table is enough for 1 x 96-well plate for each mix.

Component	Total AA Reaction Mix	DHA Reaction Mix
Assay Buffer	4.9 mL	5.0 mL
50X Enzyme Mix	100 µL	-
Blue Dye	25 µL	25 µL
Total volume	5.025 mL	5.025 mL

Δ Note: if not running a full plate, prepare the reaction mix proportionally to Total volume of AA assay (ie, for 1 mL total AA assay, use 20 µL 50X Enzyme Mix + 5 µL 200X Blue Dye + 975 µL Assay Buffer) and DHA Assay (ie, for 1 mL DHA assay, use 5 µL Blue Dye + 990 µL Assay Buffer.

13.2.2 Add 50 µL of Total AA Reaction Mix into each reaction well of the Total AA Assay plate (total volume: 100 µL/well).

13.2.3 Add 50 µL of DHA Reaction Mix into each reaction well of the DHA Assay plate (total volume: 100 µL/wel

13.2.4 Incubate plates at room temperature for 30-60 minutes, protected from light.

13.3 Measurement:

13.3.1 Measure Fluorescence increase with a fluorescence plate reader at Ex/Em = 340/430 nm (cut off: 420 nm).

14. Calculations

– Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

14.1 Average the duplicate reading for each standard and sample.

14.2 Subtract the mean fluorescence value of the blank (Standard #8) from all standard and sample readings. This is the corrected fluorescence.

14.3 Plot the corrected fluorescence values for each standard as a function of the final concentration of Ascorbic Acid (and DHA if calculating DHA/AA ratio).

14.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

14.5 Apply the corrected sample RFU reading to the standard curve to get Ascorbic Acid (B) amount in the sample wells.

14.6 Concentration of Ascorbic Acid (µM) in the test samples is calculated as:

$$\text{Ascorbic Acid concentration} = \frac{B}{V} * D$$

Where:

B = amount of total Ascorbic Acid in the sample well calculated from standard curve (µmol).

V = sample volume added in the sample wells (µL).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

14.7 Concentration of DHA (µM) in the test samples is calculated as:

$$\text{DHA concentration} = \frac{B}{V} * D$$

Where:

B = amount of DHA in the sample well calculated from standard curve (µmol).

V = sample volume added in the sample wells (µL).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

14.8 Ratio DHA/AA Determination:

Ratio = [DHA]/[total AA]

[DHA]: concentration as calculated from Step 14.7

[Total AA]: concentration as calculated from Step 14.6

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

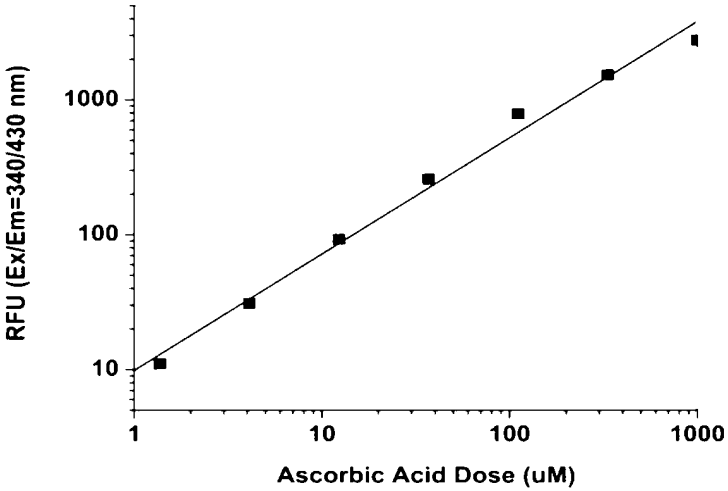


Figure 1. Typical Ascorbic Acid standard calibration curve. Ascorbic Acid dose response was measured on a solid black 96 well plate using a Gemini microplate reader (Molecular Devices). As low as 1 µM ascorbic acid can be detected with 30min incubation.

16. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare reagents and aliquot; get equipment ready.
- Prepare Ascorbic Acid standard dilution [1000-1 µM].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL) and samples (50 µL).
- Prepare Total AA Reaction Mix (and DHA reaction mix if measuring ratio):

Component	Total AA Reaction Mix	DHA Reaction Mix
Assay Buffer	4.9 mL	5.0 mL
50X Enzyme Mix	100 µL	None
Blue Dye	25 µL	25 µL
Total volume	5.025 mL	5.025 mL

- Add 50 µL of Reaction Mix into each well.
- Incubate plate at RT for 30-60 minutes.
- Measure Fluorescence increase with a fluorescence plate reader at Ex/Em = 340/430 nm (cut off: 420 nm).

17. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

18. Notes

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