ab65656

Ascorbic Acid Assay Kit (Colorimetric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Ascorbic Acid in various biological samples.

View kit datasheet: www.abcam.com/ab65656
(use www.abcam.cn/ab65656 for China, or www.abcam.co.jp/ab65656 for Japan)

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

Version 6b Last Updated 30 June 2025

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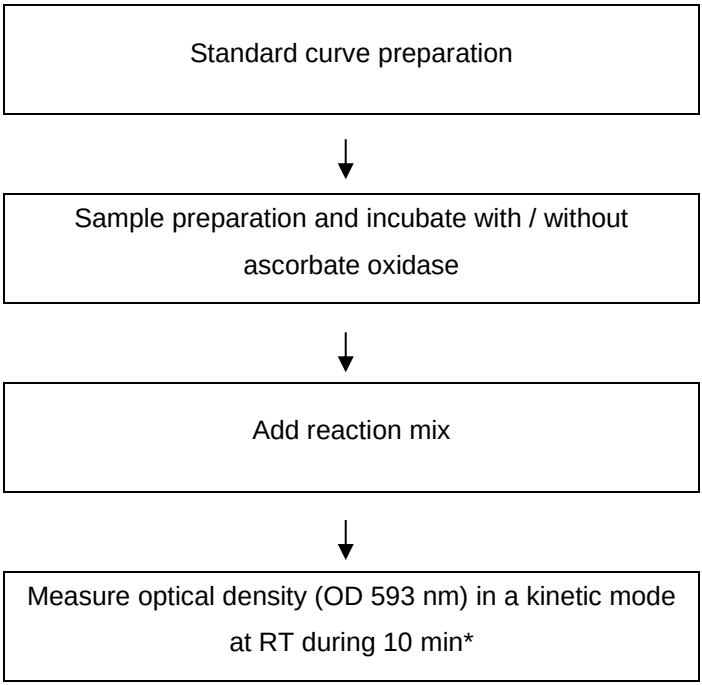
1. BACKGROUND

Ascorbic Acid Assay Kit (colorimetric) (ab65656) provides a rapid, simple, and sensitive means of detecting ascorbic acid in biological samples such as serum and other body fluids, tissue and cell extracts, growth media and food products. In this assay, Fe^{3+} is reduced to Fe^{2+} by any antioxidants present. The ferrous iron is chelated with a colorimetric probe to produce a product with a strong absorbance band which can be monitored between 545 - 600 nm. The addition of ascorbate oxidase to parallel samples removes any ascorbate present leaving a background value which is subtracted from the total to give ascorbate content. The assay can detect 0.2 to 20 nmol of ascorbic acid in various samples.

Ascorbic Acid (Vitamin C) plays an important role in many biological processes. It is a potent anti-oxidant, anti-inflammatory, anti-viral agent and an immune stimulant and is present in a wide variety of biological specimens. Due to the presence of a variety of other antioxidants in biological samples such as serum, most ascorbic acid assays show strong interference.

2. ASSAY SUMMARY



**For kinetic mode detection, incubation time given in this summary is for guidance only.*

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. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark 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 section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
FRASC buffer	25 mL	-20°C	-20°C
Ascorbic Acid probe	1 mL	-20°C	RT
Ferric Chloride Solution	1 mL	-20°C	RT
Ascorbate Oxidase	1 vial	-20°C	-20°C
Ascorbic Acid Standard	1 vial	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD = 593nm
- 96 well plate: clear plates for colorimetric assay
- Heat block or water bath
- Dounce homogenizer or pestle (if using tissue)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.

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.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- 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.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **FRASC Buffer:**

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

9.2 **Ascorbic Acid Probe:**

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot probe so that you have enough volume to perform the desired number of assays. Store at room temperature. Once opened, use within 2 months.

9.3 **Ferric Chloride Solution:**

Ready to use as supplied. Store at room temperature. Once opened, use within 2 months.

9.4 **Ascorbic Oxidase:**

Reconstitute in 500 µL ddH_2O. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months. Keep on ice while in use.

9.5 **Ascorbic Acid Standard:**

Reconstitute the Ascorbic Acid Standard (20 µmol) in 200 µL of ddH_2O to generate a 100 nmol/µL standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable. Make a fresh dilution each time.

10.1 Prepare 1 mL of 1 nmol/µL Ascorbic Acid standard by diluting 10 µL of reconstituted 100 nmol/µL standard with 990 µL of ddH_2O. Mix well.

10.2 Using 1 nmol/µL standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End [Ascorbic Acid] in well
1	0	300	100	0 nmol/well
2	6	294	100	2 nmol/well
3	12	288	100	4 nmol/well
4	18	282	100	6 nmol/well
5	24	276	100	8 nmol/well
6	30	270	100	10 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 100 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 complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 100 µL of ddH_2O.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.2 Tissue samples:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 100 µL of ddH_2O.

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.

11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.2.7 Keep on ice.

11.3 **Plasma, Serum and Urine and other biological fluids:**

Serum and plasma samples can be assayed directly.

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 Set up Reaction wells:

- Standard wells = 100 µL standard dilutions.
- Sample wells = 1 – 100 µL samples (adjust volume to 100 µL/well with ddH_2O). (This will be used to determine "total antioxidant" levels).
- Background control sample wells = 1 – 100 µL samples (adjust volume to 100 µL/well with ddH_2O). (This will be used to determine ascorbate antioxidant levels).
- To each of the Sample wells, add 10 µL ddH_2O.
- To each of the Background control sample wells, add 10 µL Ascorbate oxidase.

The set-up is summarized in the following table:

Component	Standard (µL)	Sample wells	Background control sample wells
Standard	**100**	**-**	**-**
Sample	**-**	**100**	**100**
ddH2O	**10**	**10**	**-**
Ascorbate Oxidase	**-**	**-**	**10**

12.2 Reaction Mix:

Prepare 100 µL of Reaction Mix for each reaction:

Component	Reaction Mix (µL)
FRASC Buffer	**80**
Ascorbic Acid Probe	**10**
Ferric Chloride Solution	**10**

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:
X µL component x (Number samples + standards +1).

12.3 Add 100 µL of Reaction Mix to standard and sample wells (Total antioxidant and Depleted wells).

12.4 Mix well.

12.5 Measure absorbance on a microplate reader at OD593 nm in a kinetic mode, within 2 – 3 minutes after reaction. Under these conditions, ascorbate reacts almost instantaneously with the reagent whereas other antioxidants react slowly, leading to higher background the longer the waiting time is.

***NOTE**: Wavelengths between 545 and 600 nm are acceptable as they will give 90% of the maximum absorbance.*

13. CALCULATIONS

- Subtract the value of the Background wells (containing Ascorbate Oxidase) from the wells with total antioxidants present. The difference is OD due to ascorbic acid. Determine the nmoles of ascorbate from the standard curve.

  C = ascorbate concentration in sample
  = (At – Ab)/(slope of the standard curve)/V = nmol/ml = µM

Where: **At** = Absorbance of the total antioxidant well.
Ab = Absorbance of the background well with ascorbate Oxidase.
Slope = Absorbance of 10 nmol standard – 0 nmol standard/10 nmol.
V = sample volume added into the reaction well (in ml).

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

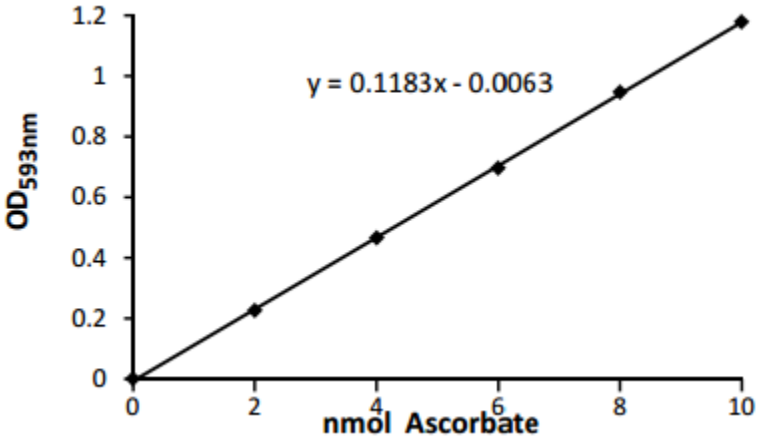


Figure 1. Typical Ascorbic Acid standard calibration curve using colorimetric reading.

15. QUICK ASSAY PROCEDURE

***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 standard, probe, Ferric Chloride Solution, enzyme (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (100 µL), samples (100 µL) and background control wells (100 µL).
- To each of the Sample wells, add 10 µL dH_2O (measure total antioxidant in sample).
- To each of the Background sample control wells, add 10 µL Ascorbate oxidase.
- Prepare Ascorbic Reaction Mix (Number samples + standards + 1).

Component	Colorimetric Reaction Mix (µL)
FRASC Buffer	**80**
Ascorbic Acid Probe	**10**
Ferric Chloride Solution	**10**

- Add 100 µL of Reaction Mix to the standard and sample wells.
- Read within 2-3 minutes.
- Measure plate at OD 593 nm for colorimetric assay.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	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	Cause	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 on 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

17. FAQ

Could results be low depending on how the samples are prepared?

Ascorbic acid is unstable and very easily oxidizable, especially if there is time lag in preparing samples for the assay. It is essential to work quickly and keep sample vials closed as much as possible to prevent air exposure. This could be the reason for low amounts of unoxidized, free ascorbic acid detected in the samples.

Does this kit distinguish between different isoforms of ascorbic acid?

No.

How sensitive is this assay?

This kit will detect ascorbic acid in the range of 0.2 – 20 nmol. However, if low levels are present in samples, Ascorbic Acid Assay (ab65346) as a fluorometric assay could be used as an alternative, which detects in the range of 0.01- 10 nmol.

18. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Chelating agents e.g. EDTA.
- Antioxidants or strong reducing agents.

19. **NOTES**

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)