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ab65329 Total Antioxidant Capacity Assay Kit (Colorimetric)

For the sensitive and accurate measurement of antioxidant proteins and/or small molecules in various biological samples.

View kit datasheet: www.abcam.com/ab65329
(use www.abcam.cn/ab65329 for China, or www.abcam.co.jp/ab65329 for Japan)

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

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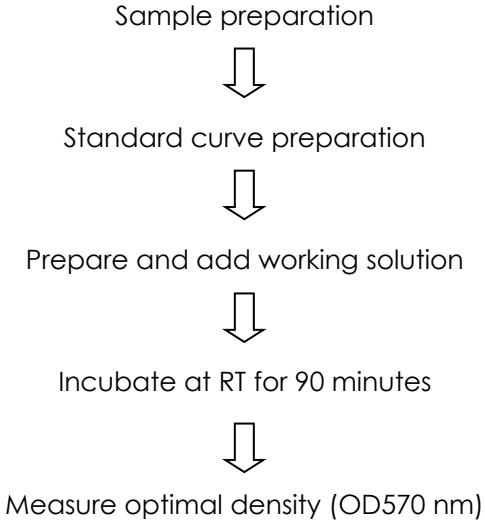
1. Overview

Total Antioxidant Capacity Assay Kit (Colorimetric) (ab65329) can measure either the combination of both small molecule antioxidants and proteins or small molecules alone in the presence of our proprietary Protein Mask. Cu^{2+} ion is converted to Cu^{+} by both small molecule and protein. The Protein Mask prevents Cu^{2+} reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu^{+} ion is chelated with a colorimetric probe giving a broad absorbance peak around OD 570 nm, proportional to the total antioxidant capacity.

Antioxidants play an important role in preventing the formation of and scavenging of free radicals and other potentially toxic oxidizing species. There are three categories of antioxidant species: enzyme systems (GSH reductase, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, vitamin E, etc.) and proteins (albumin, transferrin, etc.). Different antioxidants vary in their reducing power. Trolox is used to standardize antioxidants, with all other antioxidants being measured in Trolox equivalents.

Measurement of the combined non-enzymatic antioxidant capacity of biological fluids and other samples provides an indication of the overall capability to counteract reactive oxygen species (ROS), resist oxidative damage and combat oxidative stress-related diseases. In some cases, the antioxidant contribution of proteins is desired whereas in other cases only the contribution of the small molecule antioxidants is needed.

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 4°C 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 the Materials Supplied section.

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

Δ Note: Reconstituted components are stable for 4 months.

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 condition (before prep)	Storage condition (after prep)
Assay Diluent	10 mL	4°C	RT
Cu^{2+} Reagent	200 µL	4°C	RT
Protein Mask	10 mL	4°C	RT
Trolox Standard (1 µmol, lyophilized)	1 vial	4°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 absorbance at OD 570 nm
- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- DMSO (anhydrous)
- 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
- Dounce homogenizer (if using tissue)
- (Optional) – Triton X-100 for sample preparation

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 Diluent:

Ready to use as supplied. Equilibrate to room temperature before use. Store at room temperature.

9.2 Cu^{2+} Reagent:

Ready to use as supplied. Equilibrate to room temperature before use. Store at room temperature.

9.3 Protein Mask:

Ready to use as supplied. Equilibrate to room temperature before use. Store at room temperature.

9.4 Trolox Standard:

Reconstitute in 20 µL of pure DMSO by vortexing vial. Then add 980 µL of fdH_2O and mix to generate a 1 mM Trolox Standard solution. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Use within 4 months.

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 Use 1 mM Trolox Standard solution to prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Trolox Standard (µL)	ddH_2O (µL)	Final volume standard in well (µL)	End amount Trolox in well (nmol/well)
1	0	300	100	0
2	12	288	100	4
3	24	276	100	8
4	36	264	100	12
5	48	252	100	16
6	60	240	100	20

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 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 (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 (0.05% Triton can be used if needed).
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Incubate cell homogenate for 10 minutes on ice.
11.1.6 Centrifuge sample 2 – 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.
11.1.7 Collect supernatant and transfer to a new tube.
11.1.8 Keep on ice.

11.2 Tissue samples:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 – 100 mg).
11.2.2 Wash tissue in cold PBS.
11.2.3 Resuspend tissue in 500 – 1000 µL of ice cold PBS.
11.2.4 Homogenize tissue with a Dounce homogenizer or pestle sitting on ice, with 10 – 15 passes.
11.2.5 Incubate tissue homogenate for 10 minutes on ice.

11.2.6 Centrifuge sample 2 – 5 minutes at 4°C at top speed in a cold microcentrifuge to remove any insoluble material.
11.2.7 Collect supernatant and transfer to a new tube.
11.2.8 Keep on ice.

Δ **Note**: Sonication can be used for tissue preparation if desired.

11.3 Plasma, serum, urine and cell culture media samples:

No sample purification is required; samples can be measured directly.

Δ **Note**: For plasma, heparin is recommended.

11.4 Food samples:

11.4.1 Liquid samples: no sample preparation is required.
11.4.2 Solid samples: solid food samples should be homogenized following procedure described in step 11.2

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

12. Assay Procedure

- 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.

12.1 Cu^{2+} Working Solution:

12.1.1 Dilute 1 part of Cu^{2+} Reagent (Step 9.2) with 49 parts of Assay Diluent (Step 9.3) – for example, add 9.8 mL of Assay Diluent to the 200 µL of Cu^{2+} Reagent. Mix well.

12.2 Set up reaction wells:

SMALL MOLECULE TAC (Total Antioxidant capacity): If only measuring small molecule total antioxidant capacity, samples should be diluted 1:1 with Protein Mask (Step 9.3) prior addition to the well.

- Standard wells: 100 µL Standard dilutions.
- Sample wells = 1 – 100 µL samples (adjust volume to 100 µL/well with ddH_2O).

Δ Note: For serum, we suggest to assay 0.01 – 1 µL without Protein Mask, or 1 – 10 µL with Protein Mask.

12.3 TAC Assay:

12.3.1 Add 100 µL Cu^{2+} Working Solution to all standard and sample wells.

12.3.2 Mix and incubate plate at room temperature for 90 minutes on an orbital shaker protected from light.

12.3.3 Measure output on a microplate reader at OD 570 nm.

13. 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.

13.1 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.2 Average the duplicate reading for each standard and sample.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of Trolox.

13.4 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).

13.5 Concentration of Trolox (nmol/µL or mM) in the test samples is calculated as:

$$\text{Sample Total Antioxidant Capacity} = \left(\frac{Ts}{Sv}\right) * D$$

Where:

Ts = TAC amount in the sample well calculated from standard curve (nmol).

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

D = sample dilution factor.

14. Typical Data

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

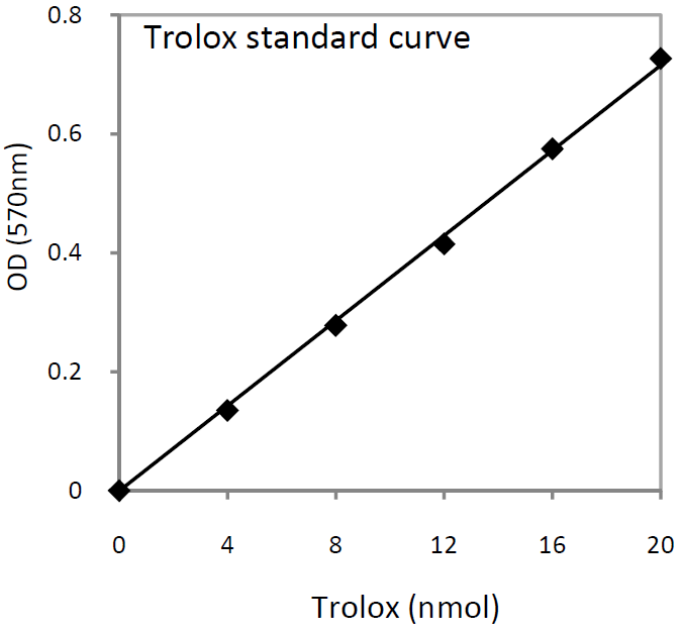


Figure 1. Typical Trolox standard calibration curve.

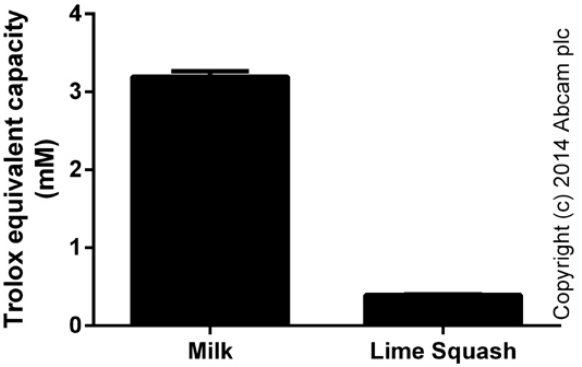


Figure 2. Trolox equivalent capacity measured in milk and concentrated squash. Background signal subtracted (duplicates ± SD).

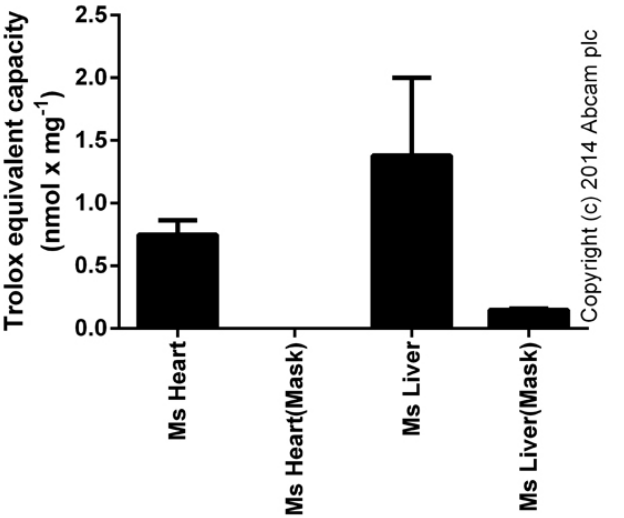


Figure 3. Trolox equivalent capacity measured in mouse tissue lysates, showing quantity (nmol) per mg of extracted protein. Results following blocking of protein activity is also shown "(Mask)" Background signal subtracted (duplicates ± SD).

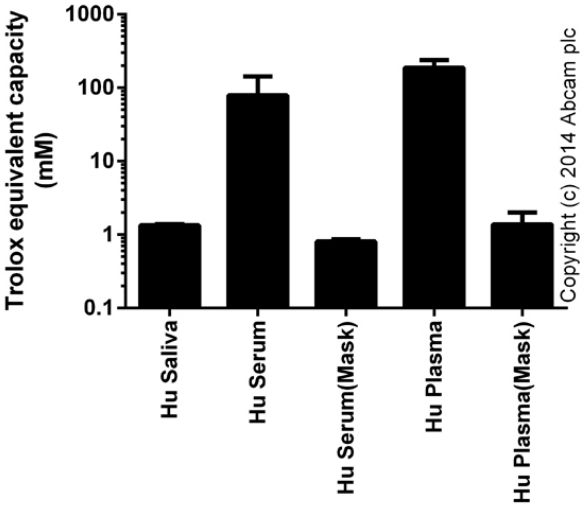


Figure 4. Trolox equivalent capacity measured in biological fluids. Results following blocking of protein activity is also shown "(Mask)" Background signal subtracted (duplicates ± SD).

15. 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.

- Solubilize Trolox standard; get equipment ready.
- Prepare Trolox standard curve [4 – 20 nmol/well]
- Prepare samples in optimal dilutions to fit standard curve readings.

  For detection of small molecule TAC: dilute samples 1:1 in Protein Mask.
- Prepare Cu^{2+} Working Solution: dilute Cu^{2+} Reagent 1:50 in Assay Diluent.
- Set up plate in duplicate for standard (100 µL) and sample (100 µL).
- Add 100 µL Cu^{2+} Working Solution to all standards and samples.
- Mix and incubate plate at RT for 90 minutes protected from light.
- Measure plate at OD 570 nm.

16. 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

17. FAQs

Q. Why do I get different results from the same sample at different times?

A. This can occur due to 3 main possibilities:

1. Different lots may give different readings. However, when using calibration curve together with the sample testing, the sample concentration should be in the same range.

2. Sample may change during storage, especially Vitamin C.

3. When using the same lot, the same sample reading should be similar, otherwise maybe some experimental error.

Q. Is the theory of TAC assay kit the same as the DPPH radical method? I am using DPPH reagent (final concentration 250 µM in toluene) and want to know is there any advantage for using your TAC assay kit.

A. The two assays are different from one another. Our TAC assay uses Cu^{++} to Cu^{+} as a mechanistic tool. It is easy to reduce so some molecules such as uric acid will respond quickly to it. Some of the disadvantages of the DPPH are:

1) This method quantifies DPPH after exposure to sample. The standard curve gives negative results.

2) Reaction kinetics between DPPH and anti-oxidants are not linear. So the antioxidant capacity is rather arbitrary.

3) The time to steady state using DPPH varies with different anti-oxidants. So you may get conflicting relative capacity depending on the reaction time.

Q. Does EDTA in blood affects the use of this kit on plasma?

A. Although small presence of EDTA should have no problem on the function of this kit, we recommend using heparin or citrate. Samples that use EDTA as anticoagulant will give a lower antioxidant capacity compared with heparinized plasma samples, but should nonetheless be sufficient for detection and data analysis.

Q. Is the protein mask necessary with urine sample, since the level of protein in urine compared to serum is minimal?

A. If the total antioxidant capacity is desired, I would not recommend the use of the protein mask. If only the levels of the small molecule antioxidants are required, please use the protein mask.

Q. Which sample is better between serum and plasma?

A. Both human serum and plasma have an antioxidant capacity of 0.5 to 2 mM. So both samples would be equally good for detection.

18. Notes

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