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Technical Datasheet

EZAssay™ Antioxidant Activity Estimation Kit (CUPRAC)

Product Code: CCK071

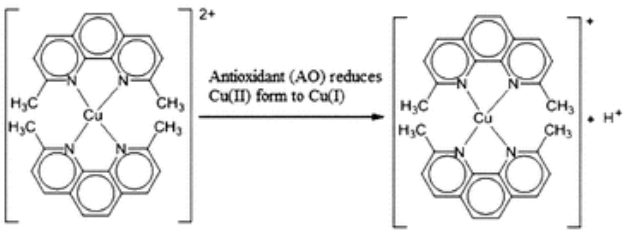
1. Introduction:

In animal cells and tissues, reactive oxygen species (ROS) are produced as a result of aerobic metabolism. These ROSs are free radicals which are highly reactive molecules having unpaired electron and gain stability by oxidizing other compounds. These free radicals generated during oxidative stress damage the biological system. This type of damage is often associated with the degenerative diseases and disorders like cancer, cardiovascular diseases and ageing.

CCK071 EZAssay™ Antioxidant Activity Estimation kit (CUPRAC) is based on ability of antioxidants in the sample to reduce copper(II)-chromogen oxidant complex. Absorbance of the reduced copper(I)-chromogen complex is indicative of antioxidant capacity of the test sample.

2. About the kit:

The method described measures the copper reducing antioxidant capacity of the test sample. This assay is based on the reduction of Cu(II)-chromogen complex into Cu(I) complex in presence of antioxidant to produce color. This complex has absorption maximum at 450nm and absorbance is directly proportional to antioxidant activity of the sample.



Cu(II) chromogen complex colored Cu(I) complex

3. Applications:

The kit can be used for estimation of antioxidant capacity of biological samples such as serum, plasma, tissue homogenates and cell lysates.

4. Kit Contents:

Contents		Quantity	Storage
Code	Description		
CCK071(A)	Tris buffer	15ml	RT
CCK071(B)	CUPRAC Developing solution	15ml	RT
CCK071(C)	Copper solution	10ml	RT
CCK071(D)	Standard Solution (Trolox) (2.5mM)	4 x 0.5ml	-20°C

**Note: Reagents supplied in the kit are sufficient for 200 tests in 96 well plate, including standards and samples.*

5. Materials required but not provided in the kit:

- Test sample (serum/ plasma/ cells/ tissue)
- Sonicator or tissue homogenizer
- Adjustable pipettes and pipette aid
- Glass test tubes
- Flat-bottom 96-well microtiter plates
- 96-well microplate reader capable of measuring absorbance between 440-460 nm OR
- Spectrophotometer capable of measuring absorbance between 440-460 nm
- Cell culture grade water/ deionized water

6. General Guidelines:

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.
- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Procedural precautions

- Do not leave the reagent bottles and sample bottles open for prolonged duration because the reagents are light sensitive and gets decomposed naturally if exposed to air for long duration. Replace the caps immediately after use.
- Colorless sample should be used to avoid assay interference as the assay is yellow color sensitive.
- Particulate matter should be removed from the sample by centrifugation or by passing the sample through 0.45 µ filter.

7. Directions for use:

Users are advised to review entire procedure before starting the assay

7.1. Preparation of reagents:

7.1.1 Chromogenic Substrate

Prepare chromogenic substrate in an amber coloured bottle as mentioned in the Table 1 given below depending on number of tests to be performed.

Note: Chromogenic substrate should be prepared just prior to performing the assay.

Table 1: Preparation of chromogenic substrate

Reagent	25 tests	50 tests	100 tests	150 tests	200 tests
CCK071(B)	1.25ml	2.5ml	5.0ml	7.5ml	10.0ml
CCK071(C)	0.5ml	1.0ml	2.0ml	3.0ml	4.0ml
CCK072(A)	0.75ml	3.0ml	3.0ml	4.5ml	6.0ml

7.2. Preparation of samples:

7.2.1. Plasma

1. Collect blood using anticoagulant.
2. Centrifuge at 904 - 1325 g for 10 minutes at 4°C.
3. Collect the top yellow plasma layer without disturbing the lower layers.
4. Store plasma on ice for immediate use. For long term storage (one month), freeze at -80°C.

7.2.2. Serum

1. Collect blood without using anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge at 2254 g for 15 minutes at 4°C.
4. Collect the top yellow serum layer without disturbing the lower layers.
5. Store serum on ice for immediate use. For long term storage (one month), freeze at -80°C.

7.2.3. Cell lysate

1. Collect 2 x 10^7 cells/ml in PBS.
2. Sonicate 3 times with 5 second intervals at 40V over ice.
3. Use whole homogenate for the assay.

7.2.4. Tissue homogenate

1. Rinse the tissue thoroughly in 1X PBS or saline to remove all traces of blood.
2. Resuspend about 20-25mg of the tissue in 200-250µl 1X PBS and sonicate for 15-20 seconds at 40V over ice.
3. Centrifuge the tissue homogenate at 3428 g for 5 minutes. Use the supernatant for the assay. Store at -80°C if not assaying on the same day.

7.3. Preparation of Standard Curve:

2.5mM Trolox standard CCK071(D) solution is provided in the kit. Serially dilute 2500µM standard solution in 1:1 ratio to obtain 1250µM, 625µM, 312.5µM, 156.25µM, 78.13 µM, 39.06 µM solution as depicted in Figure 1.

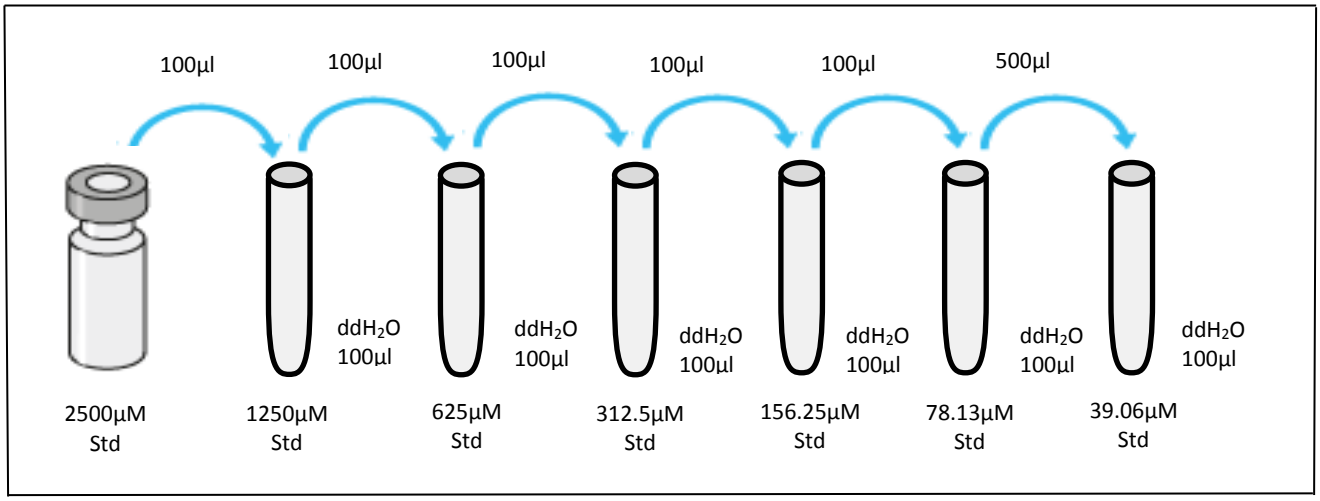


Table 2: Dilution table for Standard Trolox Solution (CCK071D) *Note: All the dilutions to be done with deionized water.*

7.4. Assay Procedure:

Optional: Instead of Trolox as standard, Ascorbic Acid can also be used. Users are advised to determine working range of standard concentration for ascorbic acid as per their assay requirement.

1. Label the 96 well plate with different concentrations of standard, control, sample and blank in triplicates.
2. Add 100µl of deionized water to each well.
3. Add 10µl sample to sample well, 10µl standard to standard well and 10µl deionized water to blank well.
4. Add 100µl chromogenic substrate (as prepared in section 6.1.1) to each well and incubate for 10 minutes in dark at room temperature.
5. Measure the absorbance at 450nm using microplate reader.

Table 2: Assay procedure

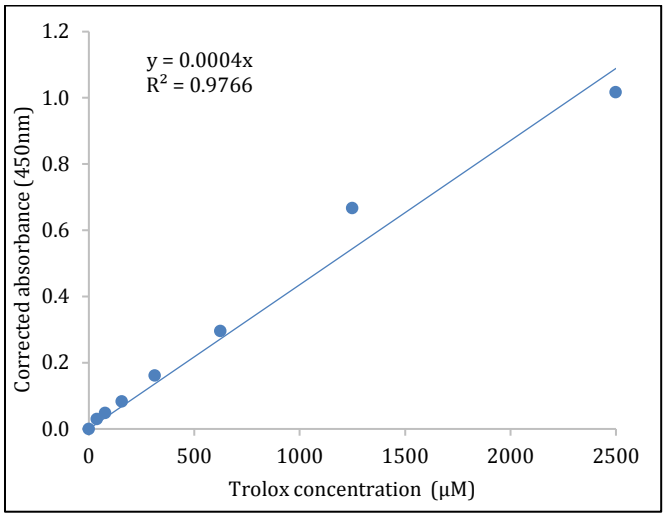
Trolox Conc^{n} (µM)	Reagent	Reagent Volume (µl)	Deionized Water (µl)	Chromo-genic Substrate (µl)
0 (Blank)	Deionized Water	10	100	100
39.6	Trolox	10	100	100
78.13	Trolox	10	100	100
156.25	Trolox	10	100	100
312.5	Trolox	10	100	100
625	Trolox	10	100	100
1250	Trolox	10	100	100
2500	Trolox	10	100	100
-	Sample	10	100	100

8. Result Analysis:

1. Determine the average values from duplicate or triplicate readings at 450nm.
2. From the value obtained in step 1 subtract the average value of blank. The value obtained is **corrected absorbance**.

Corrected absorbance = Absorbance$_{(450nm)\ (Test\ /\ standard)}$ - Absorbance$_{(450nm)\ (Blank)}$

3. Plot the corrected absorbance of Trolox standards (Y-axis) against standard Trolox concentrations (X-axis) to obtain the standard curve.
4. Given below is an example of a standard curve created by following steps described in the assay procedure.



Note: The plot given is for reference only.
***DO NOT** use it to interpret actual results.*

5. Determine slope of the standard curve (y = mx + c).
6. Calculate the Antioxidant concentration of each sample as Trolox Equivalents using the equation of the standard curve.

$$\text{Trolox Equivalents } (\mu M) = \frac{(\text{corrected absorbance}) - (\text{y - intercept})}{\text{slope}}$$

9. Storage and Shelf life:

- On receipt, store the kit components at temperatures indicated on individual labels. (Refer section 3).
- Use before the expiry date given on the product label.

10. Advantages:

- **Easy reagent preparation**: Reagents of required concentration provided, requiring only simple dilutions for reagent preparation.
- **Flexibility**: Different types of samples (serum, plasma, cells, tissue and food extracts) can be analyzed.
- **Compatibility with multiple instruments**: The absorbance can be read using a spectrophotometer or a microplate reader.

11. Troubleshooting Points:

Problems	Possible Causes	Recommended Solutions
Random absorbance values / dispersed duplicate and triplicate values	Pipetting errors	Do not splash contents in the tube or cuvette; equilibrate the pipette tips before pipetting each reagent
	Air bubbles formed in the well	Pipette gently against the wall of the well; remove the bubbles by gently tapping the side well
	Color development solution not prepared using the supplied reagents	Use only the reagents provided in the kit for preparation of color development solution
	Samples used after multiple freeze-thaw cycles	Aliquot the samples before freezing and avoid multiple free-thaw cycles
	Use of old or inappropriately stored samples	Use fresh samples or store at correct temperatures
	Use of reagents diluted previously and stored for long durations	Prepare fresh dilutions of all reagents; refer to datasheet for storage of diluted reagents
	Use of partially thawed samples	Thaw the sample completely. Equilibrate it at room temperature and mix gently before use
Readings do not follow a linear pattern for standard	Color development solution or dilutions of standard solutions stored for long duration	Prepare these reagents freshly for each assay. Do not store.
	Dilutions of standard stock not prepared correctly	Refer to the datasheet for dilutions of standards; equilibrate the pipette tips while pipetting out standard of each dilution
	Air bubbles formed in the well	Pipette gently against the wall of the tubes/cuvette/well; remove the bubbles by gently tapping the side of the tube/cuvette/well
	Calculation errors	Recheck calculations after referring to the datasheet
	Substituting reagents from older kits/lots	Only use the components given in the kit
Absorbance values higher than expected	Colored test compound being tested	Colorless sample should be used to avoid assay interference as the assay is yellow color sensitive.

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