CELLBIO™
in service of your CELL

Technical Datasheet

EZAssay™ Antioxidant Activity Estimation Kit (FRAP)

Product Code: CCK072

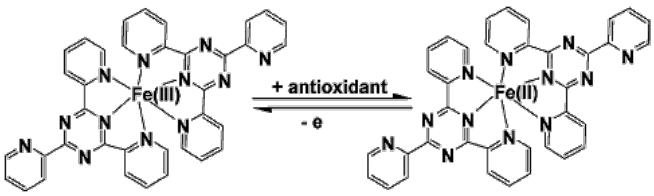
1. Introduction:

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.

CCK072 EZAssay™ Antioxidant Activity Estimation kit (FRAP) has been designed for measuring the ferric reducing ability of test sample. In this assay ferric ion-chromogen complex gets reduced to colored ferrous ion at low pH. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration.

2. About the kit:

The method described measures the ferric reducing ability of the test sample. This assay is based on the reduction Fe(III)-chromogen complex into Fe(II) at low pH in presence of antioxidant to produce intense blue color. This complex has absorption maximum at 593nm and can be measured spectrophotometrically at 560nm. Absorbance is directly proportional to antioxidant activity of the sample.



Fe(III) chromogen complex Colored Fe(II) complex

3. Applications:

Measurement of the antioxidant capacity of foods, beverages and nutritional supplements containing polyphenols.

4. Kit Contents:

Contents		Quantity	Storage
Code	Description		
CCK072(A)	Assay buffer (5X)	30ml	2 - 8°C
CCK072(B)	FRAP Reagent A	2.5ml	2 - 8°C
CCK072(C)	FRAP Reagent B	2.5ml	2 - 8°C
CCK072(D)	$FeCl_2$ standard	250mg	2 - 8°C

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

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 540-600 nm or
- Spectrophotometer capable of measuring absorbance between 540-600 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.

7. Directions for use:

Users are advised to review entire procedure before starting the assay

7.1 Preparation of reagents:

7.1.1. Assay Buffer 1X

Prepare 1X assay buffer by adding 20ml of 5X assay buffer in 80ml water. It can be used for 3 months if stored at 2-8ºC.

7.1.2. 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. Chromogenic substrate may show some undissolved particles. They can be removed by filtration through a filer paper.

Table 1: Preparation of chromogenic substrate

Reagent	25 tests	50 tests	100 tests	150 tests	200 tests
CCK072(B)	0.25ml	0.5ml	1.0ml	1.5ml	2.0ml
CCK072(C)	0.25ml	0.5ml	1.0ml	1.5ml	2.0ml
Acetate buffer 1X	2ml	4ml	8.0ml	12.0ml	16ml

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.
4. Store at -80°C if not assaying on the same day.

7.3 Preparation of Standard Curve:

1. In a clean tube, prepare10mM $FeCl_2$ stock by adding 19.8mg $FeCl_2$ powder in 10ml water.
2. Prepare 1000 µM $FeCl_2$ by adding 100 µl of stock solution in 900 µl assay buffer.
3. Serially dilute 1000µM $FeCl_2$ in 1:1 ratio to obtain 500 µM, 250µM, 125µM, 62.5µM, 31.25 µM, 15.6 µM, 7.8 µM, 3.9 µM solution as depicted in the figure below.

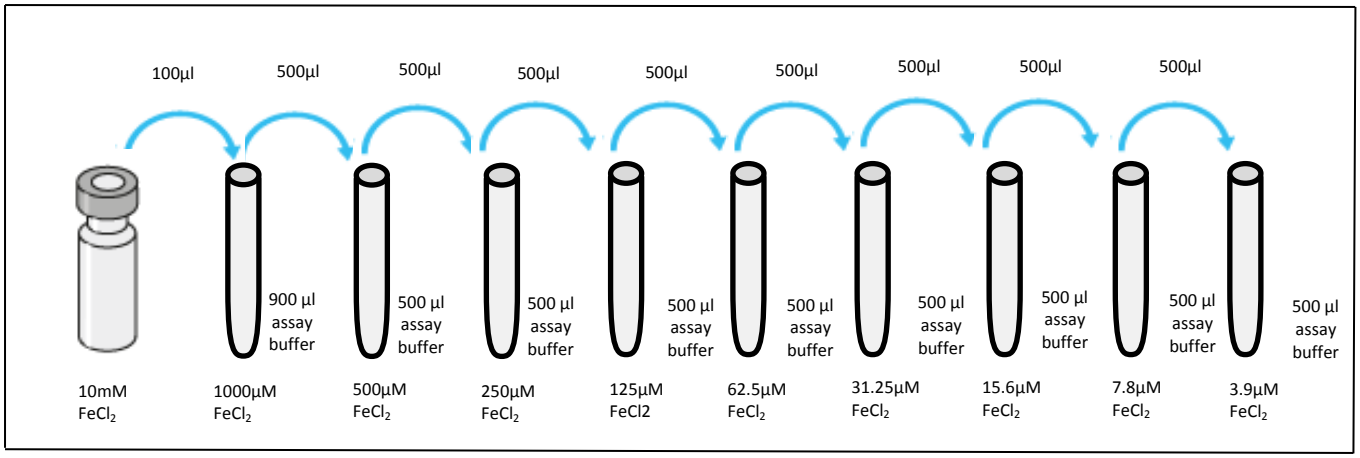


Figure 1: Dilution table for Standard Curve of $FeCl_2$

Note: All the dilutions to be done with 1X assay buffer, use 1X assay buffer as blank instead of water.

7.4 Assay Procedure

Optional: If required, Ascorbic acid, Uric acid and Trolox can be used as positive control in concentration range of 3.9 to 250µM, same as $FeCl_2$ (Refer Table 2)

1. Label the 96 well plate with different concentrations of standard, control, sample and blank.
2. Add 100µl of standard/control/sample/blank to the specified wells in triplicate.
3. Add 100µl chromogenic substrate to each well and incubate for 10 minutes in dark at room temperature.
4. Measure the absorbance at 560nm using microplate reader.

Table 2: Assay procedure

$FeCl_2$ (µM)	Reagent	Reagent Volume (µl)	Chromogenic Substrate (µl)
0 (Blank)	1X Assay buffer	100	100
3.9	$FeCl_2$	100	100
7.8	$FeCl_2$	100	100
15.6	$FeCl_2$	100	100
31.25	$FeCl_2$	100	100
62.5	$FeCl_2$	100	100
125	$FeCl_2$	100	100
250	$FeCl_2$	100	100
-	Sample	100	100

8. Result Analysis:

1. Determine the average values from duplicate or triplicate readings at 560nm and from this value, subtract the average value of blank. The value obtained is **corrected absorbance**.

   Corrected absorbance = $Absorbance_{(560nm)}$ (Test/ standard) - $Absorbance_{(560nm)}$ (Blank)

2. Plot the corrected absorbance of standards (Y-axis) against $FeCl_2$ concentrations (X-axis) to obtain the standard curve.
3. Given below is an example of a standard curve created by following steps described in the assay procedure.
   Note: The plots given here are only for reference. DO NOT use them to interpret the results.

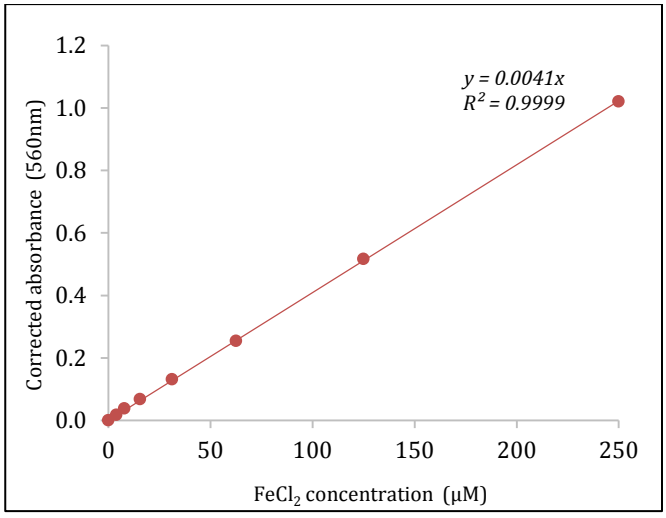


Figure 2: Standard curve of $FeCl_2$

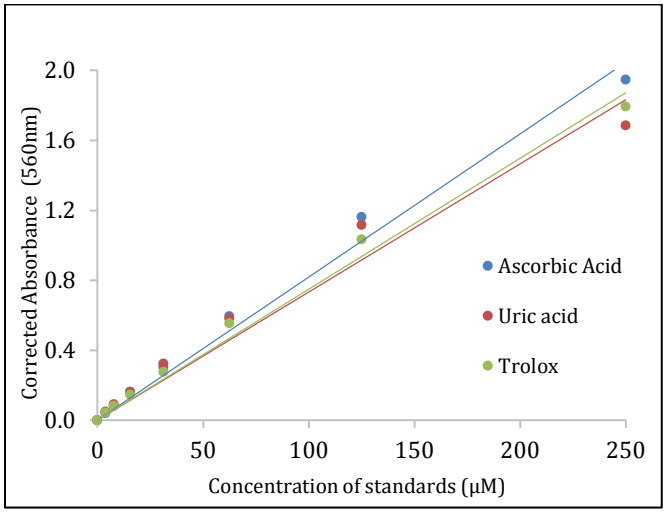


Figure 3: Standard curves of known antioxidants – Trolox, Ascorbic acid and Uric acid

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

$$\text{Fe(II) iron equivalents } (\mu M) = \frac{(\text{corrected absorbance}) - (\text{y - intercept})}{\text{slope}}$$

***Note**: If $FeCl_2$ concentration of sample is too high, dilute the sample and, multiply by the dilution factor while calculating the final concentration.*

9. Storage and Shelf life:

- On receipt, store the kit components at temperatures indicated on individual labels. (Refer section 3).
- $FeCl_2$ standard can be used for minimum 3 days at room temperature after preparation.
- 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 tubes/cuvette/well; remove the bubbles by gently tapping the side of the tube/cuvette/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
$FeCl_2$ standard not detected	$FeCl_2$ may get precipitated over time	Do not use the solution that contains precipitate.
		Use fresh solution

HiMedia Laboratories Pvt. Ltd.
Plot No. C40, Road No. 21Y, MIDC, Wagle Industrial Area, Thane (West) 400604, Maharashtra, India.Tel No.022-69034800
Email: atc@himedialabs.com Website: www.himedialabs.com.