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Technical Datasheet

EZAssay™ TBARS Estimation Kit for Lipid Peroxidation

Product Code: CCK023

1. Introduction:

Lipid peroxidation can be defined as the oxidative degradation of lipids by reactive oxygen species. Since lipids form a major component of cell membranes, lipid peroxidation leads to breakdown of the membrane structure and leakage of cellular components. Also, the products of lipid peroxidation (lipid peroxides) and their derivatives inhibit protein synthesis, alter enzyme activity and damage nucleic acids.

In animal cells and tissues, oxidative stress is generated due to several reasons such as cellular injury, aging, inflammation, tumorigenesis etc. Oxidative stress is associated with many pathophysiological conditions like atherosclerosis, cardiovascular disease, diabetes, liver disorder, inflammatory diseases and degenerative diseases.

During oxidation, reactive oxygen species degrade cellular lipids into lipid peroxides that serve as indicators of oxidative stress in cells and tissues. However, these lipid peroxides are extremely unstable, have very short half-lives and cannot be measured directly. The most commonly used method for estimating lipid peroxidation in the cells is, measurement of by-products, the low-molecular weight thiobarbituric acid reactive substances (TBARS) generated as a result of decomposition of lipid peroxides. Melondialdehyde (MDA) is one of such TBARS used for estimation of lipid peroxidation.

2. About the kit:

This assay is based on the reaction of MDA with a chromogenic agent thiobarbituric acid (TBA) at high temperature and acidic conditions to form MDA-TBA adduct (1:2). This complex has absorption maximum at 532nm and can be measured spectrophotometrically between 530 - 540nm. Absorbance is directly proportional to the TBARS concentration in the sample.

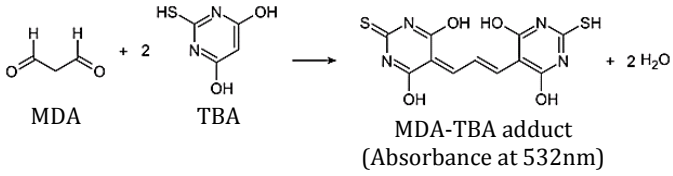


Figure 1: Reaction between MDA and TBA to form the MDA-TBA adduct

The components of one kit are sufficient to perform 100 tests including controls, standards and samples.

3. Applications:

- Estimation of lipid peroxidation in cells treated with oxidizing agents or test drugs
- Estimation of lipid peroxidation in serum, urine and tissues of animals treated with oxidizing agents or test drugs

4. Kit Contents:

Contents		Quantity	Storage
Code	Description		
CCK023 (Part F)	Thiobarbituric acid	270mg	15-30°C
CCK023 (Part G)	Acid reagent	3 × 25ml	15-30°C
CCK023 (Part H)	500µM Malondialdehyde	1ml	2 - 8°C

5. Materials required but not provided in the kit:

- Test sample (serum/plasma/cells/tissue)
- Concentrated hydrochloric acid
- Adjustable pipettes and pipette aid
- Glass test tubes
- Flat-bottom 96-well microtiter plates or
- Quartz cuvettes

- 96-well microplates reader capable of measuring absorbance between 530-540nm or
- Spectrophotometer capable of measuring absorbance between 530-540nm
- Cell Culture Grade 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 lipid peroxides in the test samples decompose 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

1. Preparation of reagents:

0.25N Hydrochloric acid (0.25 HCl)

Prepare 1N HCl solution by adding 850µl of conc. HCl in 9150µl of distilled water. Mix 1 part of 1N HCl with 3 parts of distilled water to obtain 0.25N HCl. (2.5ml of 1N HCl + 7.5ml of distilled water).

TBA solution

Weigh 37.5mg of TBA and add in a glass beaker containing 10ml of distilled water. TBA is sparingly soluble in water.

Color Development Solution

Mix 0.25N HCl, TBA solution and acid reagent (CCK023(G)) in 1:1:1 ratio. Swirl the solution vigorously to dissolve TBA powder. Cover the beaker with aluminium foil and keep in a water bath at 37°C until TBA powder is completely dissolved. Swirl the beaker intermittently to facilitate dissolution. 30ml colour development solution is sufficient for 15 tests.

Note: Prepare the color development solution as per requirement. Do not store.

2. Preparation of samples:

Plasma

1. Collect blood using anticoagulant.
2. Centrifuge at 1900 - 2300rpm for 10 minutes at 4°C.
3. Collect the top yellow plasma layer without disturbing the lower layers.
4. Store plasma on ice. For long term storage (one month), freeze at -80°C.

   Note: Normal human serum has peroxide level of 1.86 - 3.94µM expressed in terms of MDA[1]

   To minimize haemoglobin interference, prepare plasma sample as soon as possible after collecting the blood.

Serum

1. Collect blood without using anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge at 3000rpm for 15 minutes at 4°C.
4. Collect the top yellow serum layer without disturbing the lower layers.
5. Store serum on ice. For long term storage (one month), freeze at -80°C.

   Note: Normal human serum has peroxide level of 1.86 - 3.94µM expressed in terms of MDA[1]

Urine

1. Centrifuge the collected urine at 7500g for 5 minutes to remove insoluble particles.
2. Use the supernatant for the assay.
3. Store at -80°C if not assaying on the same day.

   Note: Normal human urine has a lipid peroxide level (expressed in terms of MDA) of 0.8-2.0µmol/g creatinine[2]

Cell lysate

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

Tissue homogenate

1. Rinse the tissue thoroughly in 1X PBS or saline to remove all traces of blood.

   Note: Hemoglobin interferes with the assay.
2. Resuspend about 20-25mg of the tissue in 200-250µl 1X PBS and sonicate for 15-20 seconds at 40V setting over ice.
3. Centrifuge the tissue homogenate at 5000rpm for 5 minutes. Use the supernatant for the assay.
4. Store at -80°C if not assaying on the same day.

3. Preparation of Standard Curve:

In clean glass test tubes prepare the dilutions of 500µM MDA standard in range of 2 - 10µM. Refer table 1 for more details.

Table 1: Dilution table for Standard Curve of MDA

MDA concentration	Quantity of 500µM MDA stock	Diluent (HPLC-grade water)
2 µM	4 µl	996 µl
4 µM	8 µl	992 µl
6 µM	12 µl	988 µl
8 µM	16 µl	984 µl
10 µM	20 µl	980 µl

4. Assay Procedure:

Table 2: Assay procedure

	Blank 0µM	2µM	4µM	6µM	8µM	10µM	Sample
MDA Standard solution	-	100 µl	100 µl	100 µl	100 µl	100 µl	-
Sample	-	-	-	-	-	-	100µl
Water*	100µl	-	-	-	-	-	-
Color develop-ment solution	2ml	2ml	2ml	2ml	2ml	2ml	2ml

Note: For cell lysates and tissue homogenates suspended in PBS, use PBS as blank instead of water.

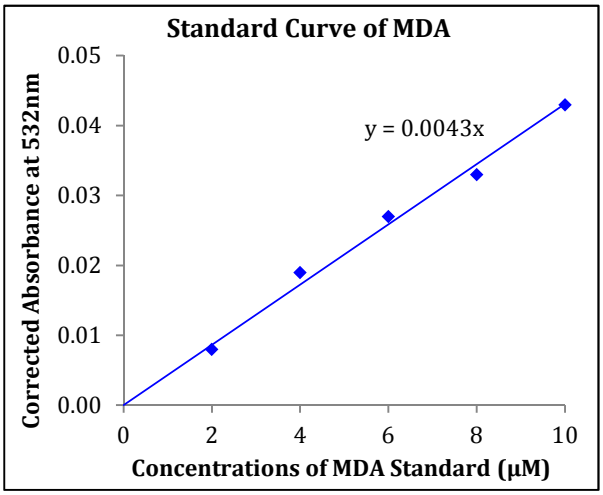
1. In clean and labeled glass tubes, add reagents, samples and standard MDA solutions as given in the table 2.
2. Cover the mouth of the tubes with aluminium foil (to prevent evaporation) and place them in boiling water bath for 60 minutes.
3. After 60 minutes, place the tubes immediately in ice bath for 10 minutes.
4. Centrifuge the tubes at 3000rpm for 10 minutes at 4°C.
5. Keep the tubes at room temperature for 30 minutes.
6. If measuring using microplate reader, take out 150µl sample from each tube in a 96-well plate.
7. If measuring using a spectrophotometer, take out required amount (depending on the size of the cuvette) of sample from each tube in a cuvette.
   Note: Wipe the external surface of cuvettes with lint-free tissue paper to minimize the handling error.
8. Measure the absorbance at 530-540nm.

8. Result Analysis:

1. Determine the average values from duplicate or triplicate readings at 530 - 540nm and from this value, subtract the average value of blank. The value obtained is **corrected absorbance**.

   Corrected absorbance = $\text{Absorbance}_{(530\text{-}540\text{nm})\ (\text{Test / standard})}$ - $\text{Absorbance}_{(530\text{-}540\text{nm})\ (\text{Blank})}$

2. Plot the corrected absorbance of standards (Y-axis) against MDA 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 plot given below is for reference only. **Do not** use it to interpret actual results.*



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

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

Note: If MDA concentration of sample is too high, either dilute the sample, or prepare standards of higher concentrations. If diluting the sample, 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).
- Do not allow the MDA standard stock to remain at room temperature for long periods of time.
- 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, cells and tissue) 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 tube/ cuvette/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	Colour 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 tube/ cuvette/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
MDA not detected in the sample	MDA concentration in sample is extremely low or sample is very dilute	In case of plasma, urine and serum, do not dilute the sample
		In case of tissue - Prepare concentrated tissue homogenate by sonicating more amount of tissue (50 - 60mg) in 200 - 250µl PBS.
		In case of cells - Prepare concentrated cell lysate by harvesting more cells.

12. References:

1. Yagi K. Simple assay for the level of total lipid peroxides in serum or plasma. Methods in Molecular Biology 108, 101-106 (1998)
2. Fatima MN, Vivek AS, Amreeta D, Uma DP. Oxidative stress and antioxidant status in primary bone and soft tissue sarcoma. BMC Cancer 2011, 11:382