Malondialdehyde (MDA) test kit

(Cat/No.: BC021 Size: 100T/96S TBA method)

This kit is an improved version of numerous domestic and international testing methods, offering a micro-volume, rapid, simple, accurate, and harmless testing method for operators . The test can reflect the levels of lipid peroxides at the cellular and subcellular levels in serum (plasma), extracellular fluids such as milk, erythrocytes, leukocytes, cultured cells, and various animal and plant cells.

1. Significance of the measurement

The body generates oxygen free radicals through both enzymatic and non-enzymatic systems. These free radicals attack polyunsaturated fatty acids (PUFAs) in biological membranes, triggering lipid peroxidation and forming lipid peroxides. Examples of free radicals include aldehydes (malondialdehyde, MDA), ketones, hydroxyl groups, carbonyl groups, hydroperoxides, or endoperoxides, as well as new oxygen free radicals. Lipid peroxidation not only converts reactive oxygen species (ROS) into reactive chemical agents—non-radical lipid breakdown products—but also amplifies their effects through chain or branched chain reactions. Therefore, an initial ROS can lead to the formation of numerous lipid breakdown products; some are harmless, while others can cause cellular metabolic and functional disorders, even cell death. Oxygen free radicals cause cell damage not only through the peroxidation of polyunsaturated fatty acids (PUFAs) in biological membranes but also through the breakdown products of lipid hydroperoxides. Therefore, the amount of MDA tested can often reflect the degree of lipid peroxidation in the body , indirectly reflecting the degree of cell damage.

The measurement of MDA is often combined with the measurement of SOD. The level of SOD activity indirectly reflects the body's ability to scavenge oxygen free radicals, while the level of MDA indirectly reflects the severity of free radical attack on the body's cells. The analysis of SOD and MDA results is helpful for the development of medicine, biology, pharmacology and industrial and agricultural production.

2. Measurement principle

Malondialdehyde (MDA), a product of lipid peroxidation degradation, can condense with thiobarbituric acid (TBA) to form a red product with a maximum absorption peak at 532 nm. Because the substrate is thiobarbituric acid (TBA), this method is called the TBA method.

3. Required Instruments and reagents required

Visible spectrophotometer or ELISA reader, constant temperature water bath or boiling water bath adjustable to about 95℃, centrifuge, vortex mixer, 10mL centrifuge tubes, distilled water, anhydrous ethanol (small amount), glacial acetic acid (analytical grade, concentration ≥99.5%), 50% glacial acetic acid (can be obtained by diluting glacial acetic acid 1:1 with distilled water), chloroform, distilled water, physiological saline, etc.

4. Sample Pretreatment

Liquid samples such as serum (plasma), myocardial perfusion fluid, renal dialysis fluid, and cell culture medium: direct detection (for some aquatic animals with high serum MDA content, a pre-test (measuring OD value less than 0.4) is required before measurement);

Animal tissue samples: Accurately weigh the animal tissue and add 9 volumes of homogenizing medium (physiological saline or 0.01 mol/L PBS) at a weight (g):volume (mL) ratio of 1:9. Homogenize mechanically under ice-water bath conditions to prepare a 10% homogenate. Centrifuge at 3500 rpm for 10 minutes and collect the supernatant for testing. (Some aquatic animals or shrimp/crab hepatopancreas may have higher MDA content; a preliminary test (OD value less than 0.4) is required before batch experiments.) (The protein concentration of this homogenate supernatant needs to be determined; protein assay kits are available from our company.)

Plant tissue samples: Accurately weigh the plant tissue and add 4 times the volume of homogenizing medium (recommended 0.01 mol/L pH 7.0－7.4 phosphate buffer) at a weight (g): volume (mL) ratio of 1:4. Mechanically homogenize under ice-water bath conditions to prepare a 20% homogenate. Centrifuge at 3500–4000 rpm for 10 minutes and collect the supernatant for analysis (some medicinal materials or plant powders can be processed using the same method).

Cell samples: After collecting cells, add 0.2-0.3 mL of physiological saline (or 0.01 mol/L PBS) to each cell sample (the number of cells should not be less than 106, the more the better). Sonicate the cells in an ice-water bath (power 200-300W, run for 5 seconds, interval 15 seconds, repeat for 2-5 minutes). Do not centrifuge and directly aspirate for analysis (the protein concentration of the homogenate needs to be measured (protein assay kits are available from our company) or the cells should be counted before homogenization).

Red blood cell samples: For processing and handling methods, please refer to point 10 of this instruction manual;

Note: If the tissue sample is a high-fat or oily sample and only MDA is measured, anhydrous ethanol can be used as the homogenizing medium (but care should be taken to seal it

during homogenization to prevent ethanol evaporation from causing inaccurate results). In this case, protein measurement is not required in the homogenate, and the fresh weight of the tissue is used for calculation.

5. Composition and Preparation: (Reagent kit shelf life: 6 months)

Reagents	50 tubes/48 samples	100 tubes /96 samples	Storage conditions
Reagent 1	10mL bottle of liquid	20mL bottle of liquid	4 ℃ or room temperature
Reagent 2	6mL bottle of liquid	12 mL bottle of liquid	4 ℃
	Add 170mL of distilled water to each bottle and mix well , or prepare according to the ratio (6:170). Store at 4 ℃ for 3-6 months.	Add 340 mL of distilled water to each bottle and mix well , or prepare according to the ratio (6:170). Store at 4 ℃ for 3-6 months.	4 ℃
Reagent 3	Powder x 1 tube	Powder x 1 tube	4 ℃
	To use, add 30 mL of distilled water to the powder and heat to 90-100 ° C to dissolve . After slightly cooling, add 30 mL of glacial acetic acid and mix. Store at 4 ° C for 3-6 months.	To use, add 60 mL of distilled water to the powder and heat to 90-100 ° C to dissolve . After slightly cooling, add 60 mL of glacial acetic acid and mix. Store at 4 ° C for 3-6 months.	4 ℃, protected from light
Standard products	10 nmol/mL tetraethoxypropane 5 mL × 1 bottle	10 nmol/mL tetraethoxypropane 5 mL × 1 bottle	4 ℃

Note: When preparing reagent 3, if using an open container such as a beaker, add 2-4 mL more water to account for evaporation loss during heating; the reagent will solidify when cooled, so it can be heated to 37℃ before each test to accelerate dissolution until it becomes transparent before use .

6. Operating steps

1. Standard Operating Procedures:

	Blank well	Standard well	Determination well	Control well
10 nmol/mL standard (mL)		0.1		
Anhydrous ethanol (mL)	0.1			
Test sample (mL)			0.1	0.1
Reagent 1 (mL)	0.1	0.1	0.1	0.1
Mix well (shake the centrifuge tube rack a few times).				
Reagent 2 (mL)	3	3	3	3
Reagent 3 (mL)	1	1	1	
50% glacial acetic acid (mL)				1
Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95 ° C water bath for 40 minutes, remove and cool in cold water , then centrifuge at 3500–4000 rpm for 10 minutes (centrifugation time should be extended below 3000 rpm to ensure complete precipitation . If a large amount of lipids is present, add 0.1 mL of				

chloroform, vortex for about 60 seconds, then centrifuge) . Collect the supernatant, and measure the OD value of each tube at 532 nm with a 1 cm optical path length using a spectrophotometer (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .

Note: ① Generally, only 1 to 2 standard tubes and blank tubes need to be tested per batch . If the sample does not have special color, turbidity or other phenomena, the control tube can be omitted and the blank tube can be used to replace the control tube for calculation .

② When aspirating the supernatant for colorimetric analysis , do not aspirate the precipitate at the bottom. If solid matter remains in the supernatant after centrifugation, increase the centrifugation speed or add 0.1 mL of chloroform, vortex for 60 seconds, centrifuge again, and then aspirate the supernatant.

③ When reading the microplate reader, simply add 200 μL of supernatant to a 96-well plate and read the value at 532 nm .

2. Reference Sampling Concentration (Volume): Except for aquatic animal samples where the sampling concentration needs to be determined in advance, serum from other animals is generally sampled directly at 0.1 mL according to the procedure table (or even 0.2 mL or 0.3 mL if the sample volume is sufficient). Tissue homogenates are generally sampled at a concentration of 10% (liver samples require slightly higher concentrations, so 0.1 mL can be taken directly; other tissues can be sampled at 0.2 mL or 0.3 mL if the volume is sufficient). Cell homogenates or cell culture media can be sampled at 0.3-0.5 mL (the recommended cell count is above 10^6). (When increasing the sampling volume, the amounts of anhydrous ethanol in the blank tube, standard in the standard tube, and reagent 1 should also be increased accordingly; other reagent volumes remain unchanged.)

3. Reference OD of standard tube: When operating according to the operation table, the OD of the standard tube minus the OD of the blank tube is 0.065-0.070 (when measuring with an ELISA reader, the reading is about 0.04 when taking 200μL, and this value will increase as the sample amount of the standard increases).

4. If the OD of the test sample is found to be too low, the water bath time can be extended from 40 minutes to 80 minutes. However, the MDA test in the same project must be extended to 80 minutes to avoid batch-to-batch differences.

5. Calculation formula:

(1) Formula for calculating MDA content in serum (plasma):

$$\text{MDA content in serum (plasma) (nmol/mL)} = \frac{\text{Determination OD}-\text{Control OD}}{\text{Standard OD}-\text{Blank OD}} \times \text{Standard concentration (10nmol/mL)} \times \text{Dilution factor before sample test}$$

(2) Formula for calculating MDA content in tissues based on protein concentration :

$$\text{MDA content in tissue (nmol/mgprot)} = \frac{\text{Determination OD}-\text{Control OD}}{\text{Standard OD}-\text{Blank OD}} \times \text{Standard concentration (10nmol/mL)} \div \text{Tissue protein concentration (mgprot/mL)}$$

(3) The formula for calculating the MDA content in tissues based on sample fresh weight is as follows :

$$\text{MDA content in tissue (nmol/tissue)} = \frac{\text{Determination OD}-\text{Control OD}}{\text{Standard OD}-\text{Blank OD}} \times \text{Standard concentration (10nmol/mL)} \div \frac{\text{Tissue weight (g)}}{\text{Homogenate total volume (mL)}}$$

(4) Formula for calculating MDA content in cells:

$$\text{MDA content in cells (nmol/}10^4\text{cells)} = \frac{\text{Determination OD}-\text{Control OD}}{\text{Standard OD}-\text{Blank OD}} \times \text{Standard concentration (10nmol/mL)} \div \frac{\text{Cell count (}10^4\text{cells)}}{\text{Homogenate total volume (mL)}}$$

6. Calculation Example:

Example 1: Take 0.1 mL of a certain serum sample and measure the MDA content. The OD of the test tube is 0.036, the OD of the standard tube is 0.076, and the OD of the standard blank tube is 0.006. The control tube is replaced with a standard blank tube. The calculation results are as follows:

$$\text{MDA content in serum (plasma) (nmol/mL)} = \frac{0.036-0.006}{0.076-0.006} \times 1 \times 4 = 4.29\text{nmol/mL}$$

Example 2: Take 0.1 mL of egg yolk, add 0.9 mL of anhydrous ethanol, mix thoroughly, extract for 3 minutes, centrifuge at 4000 rpm for 10 minutes, and collect 0.2 mL of the supernatant to detect the MDA content. The OD of the test tube is 0.198, the OD of the standard tube is 0.151, and the OD of the standard blank tube is 0.009. The control tube is replaced with a standard blank tube. The calculation results are as follows:

$$\text{MDA content in egg yolk (nmol/mL)} = \frac{0.198-0.009}{0.151-0.009} \times 10 \times 10 = 133.098\text{nmol/mL}$$

Example 3: 0.1 mL of 10% rat liver tissue homogenate was taken to detect MDA content. The OD of the test tube was 0.214, the OD of the standard tube was 0.079, and the OD of the standard blank tube was 0.009. The control tube was replaced with a standard blank tube. The protein concentration of 10% rat liver homogenate was 8.5 mg prot/mL. The calculation results are as follows:

$$\text{MDA content in rat liver (nmol/mgprot)} = \frac{0.214-0.009}{0.079-0.009} \times 10 \div 8.5 = 3.45\text{nmol/mgprot}$$

Example 4: Take 0.1 mL of 10% carp brain tissue homogenate and test the MDA content. The OD of the test tube is 0.314, the OD of the standard tube is 0.076, and the OD of the standard blank tube is 0.006. The control tube is replaced by the standard blank tube. The protein concentration of 10% carp brain tissue homogenate is 5.0788 mg prot/mL. The calculation is as follows:

$$\text{MDA content in crucian carp muscle (nmol/mgprot)} = \frac{0.314-0.006}{0.077-0.006} \times 10 \div 5.0788 = 8.603\text{nmol/mgprot}$$

Example 5: Take 0.1 mL of 20% earthworm tissue homogenate and test the MDA content. The OD of the test tube is 0.082, the OD of the standard tube is 0.074, the OD of the standard blank tube is 0.005, and the OD of the control tube is 0.025. The protein concentration of 20% earthworm tissue homogenate is 10.3145 mg prot/mL. The calculation is as follows:

$$\text{MDA content in silkworm pupa (nmol/mgprdt)} = \frac{0.082-0.025}{0.074-0.005} \times 10 \div 10.3145 = 0.8009\text{nmol/mgprdt}$$

Example 6: Take 0.2 mL of 10% rice leaf homogenate and test the MDA content. The OD of the test tube is 0.077, the OD of the standard tube is 0.140, and the OD of the standard blank tube is 0.003. The control tube is replaced by the standard blank tube. The protein concentration of 10% rice leaf homogenate is 3.2729 mg prot/mL. The calculation results are as follows:

$$\text{MDA content in rice leaf (nmol/mgprot)} = \frac{0.077-0.003}{0.140-0.003} \times 10 \div 3.2729 = 1.6504\text{nmol/mgprot}$$

Appendix I: Simplified Operating Method

(This method is more convenient if the sample size is large .)

1. Preparation of mixed reagents:

ratio of working solution I is: Reagent 1 : Reagent 2 : Reagent 3 = 0.1 : 3 : 1. Prepare and use immediately.

ratio of working solution II is: reagent I: reagent II: 50% glacial acetic acid = 0.1 :3:1. Prepare and use immediately.

2. Simple Operation Table:

	Blank well	Standard well	Determination well	control well
10 nmol/mL standard (mL)		0.1		
Anhydrous ethanol (mL)	0.1			
Test sample (mL)			0.1	0.1
Working solution I (mL)	4	4	4	
Working solution II (mL)				4

Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95 °C water bath for 40 minutes, remove and cool in cold water , then centrifuge at 3500–4000 rpm for 10 minutes (centrifugation time should be extended below 3000 rpm to ensure complete precipitation). Take the supernatant at 532 nm (1 cm optical path), zero the plate with distilled water, and measure the OD value of each tube (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .

★ **Please refer to the standard operating procedure on page 5 for important notes.**

★ **The calculation formula is the same as the standard operating procedure.**

Appendix II: Micro-volume handling methods

For samples with low concentrations, such as mouse serum (plasma), pediatric serum (plasma), mouse lung tissue, skin tissue, kidney tissue, retina, cells, and cell culture medium, the following methods can be used for detection .

(—) It is best not to centrifuge the prepared tissue homogenate . When taking a sample, make sure to shake it well and take the sample immediately.

(二) Reagent Preparation:

1. The preparation of reagents in the micro-operation method is as follows:

① Prepare reagent solution 3 according to the standard operating procedure : Dissolve one 100-tube/96-sample MDA No. 3 powder in 64mL of distilled water (or 32mL of distilled water for 50-tube/48-sample No. 3 powder) by heating , then add 60mL of glacial acetic acid (or 30mL for 50-tube/48-sample No. 3 powder) and mix well .

② Next, dilute the prepared reagent solution three above with 50% glacial acetic acid (50% glacial acetic acid is prepared by mixing 50mL of distilled water with 50mL of glacial acetic acid) at a ratio of 2:1. Prepare only the amount needed. Store the prepared reagent in a light-proof refrigerator at 4°C (providing your own glacial acetic acid).

[Note]: Because distilled water will evaporate during the heating process , 60mL or 30mL should be added , but now 4mL or 2mL is added to account for evaporation loss .

(三) Operating Procedures:

	Blank well	Standard well	Determination well	Control well
10 nmol/mL standard (mL)		0.1		
Anhydrous ethanol (mL)	0.1			
Test sample (mL)			0.1	0.1
Reagent 1 (mL)	0.1	0.1	0.1	0.1
Mix well (shake the centrifuge tube rack a few times).				
Reagent 2 application solution (mL)	1.5	1.5	1.5	1.5
Reagent 3 solution (mL)	1.5	1.5	1.5	
50% glacial acetic acid (mL)				1.5

Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95°C water bath for 40 minutes, remove and cool in cold water, then centrifuge at 3500–4000 rpm for 10 minutes (centrifugation time should be extended below 3000 rpm to ensure complete precipitation). Take the supernatant at 532 nm (1 cm optical path), zero the plate with distilled water, and measure the OD value of each tube (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .

Note : ① (For samples with low content) If the sample volume is sufficient, the sampling volume can be increased to 0.2 mL (at the same time, 0.2 mL of anhydrous ethanol, standard, and reagent 1 are added, while the volumes of other reagents remain unchanged) .

② : The OD values of each tube measured using this method are higher than those measured using the standard operating method, but this does not affect the final result.

③ : If the OD of the test tube is found to be too low, the water bath time can be extended from 40 minutes to 80 minutes. However, the MDA test in the same project must be extended to 80 minutes to avoid batch-to-batch differences.

④ : The reference OD for the standard tube in this method is: when the standard sample volume is 0.1 mL, the OD of the standard tube minus the OD of the blank tube is 0.103 to 0.112 . This value will increase as the standard sample volume increases.

(四) Calculation formula and examples:

1. Refer to the standard operating procedure on page 7 for the calculation formula.

2. Calculation Example:

Example 1: Take 0.1 mL of undiluted serum from a person and test the MDA content. The OD of the test tube is 0.030, the OD of the standard tube is 0.104, and the OD of the standard blank tube is 0.009. The control tube is replaced with a standard blank tube. The calculation is as follows:

1. MDA Content in Human Blood

$$\text{MDA (nmol/mL)} = \frac{0.030-0.009}{0.104-0.009} \times 10 \times 1 = 2.21$$

Example 2: Take 0.1 mL of 5% rat lung tissue homogenate and detect MDA content. The OD of the test tube is 0.062, the OD of the standard tube is 0.104, and the OD of the standard blank tube is 0.009. The control tube is replaced with a standard blank tube. The protein concentration of 5% rat lung tissue homogenate is 2.45 mg/mL. The calculation is as follows:

2. MDA Content in Rat Lung

$$\text{MDA (nmol/mgprot)} = \frac{0.102-0.009}{0.064-0.009} \times 10 \div 2.45 = 2.280$$

Appendix III: Determination of MDA in High-Fat Oils or Fats

(For example, the amount of MDA in soybean oil, salad oil, or rapeseed oil can be determined using the following methods .)

1. Operating Instructions:

	Blank well	Standard well	Determination well
10 nmol/mL standard (mL)		0.1	
Anhydrous ethanol (mL)	0.1		
Oily or fatty samples or high-lipid serum (plasma) (mL)			0.1
Reagent 1 (mL)	0.1	0.1	0.1
Reagent 2 application solution (mL)	3.0	3.0	3.0
Reagent 3 solution (mL)	1.0	1.0	1.0
Cover the centrifuge tubes, poke a small hole in the cover with a needle , vortex to mix, and incubate in a 95 ℃ water bath (or boil uncovered) for 80 minutes. Remove and cool with cold water.			
Chloroform (mL)	0.1	0.1	0.1
Vortex for 1-3 minutes, then centrifuge at 3500-4000 rpm for 10 minutes . Collect the supernatant and measure the OD value of each tube at 532 nm with a 1 cm optical path length using distilled water as the zeroing point (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .			

Note: ① Generally, only 1 to 2 standard tubes and blank tubes need to be made per batch .

②**Oil** and fat samples generally need to be diluted 2-3 times with anhydrous ethanol before sampling and testing . Some high-lipid serum (plasma) samples need to be diluted 2 times with physiological saline before testing.

③ When aspirating the supernatant for colorimetric analysis, avoid aspirating the bottom precipitate and chloroform. When reading the value using an ELISA reader, simply aspirate 200 μL of supernatant and add it to the 96-well plate, then read the value at 532 nm .

2. Reference OD of standard tube: The OD of the standard tube minus the OD of the blank tube is 0.065 to 0.070 when measured by spectrophotometer (approximately 0.045 when measured by ELISA reader with 200 μL sample ; this value will increase as the sample amount of standard increases) .

3. Calculation formula and examples:

1. MDA Content in Hyperlipidemic Blood (nmol/mL)

Formula:

$$\text{MDA (nmol/mL)} = \frac{\text{Determination OD}-\text{Control OD}}{\text{Standard OD}-\text{Blank OD}} \times \text{Standard Concentration (10nmol/mL)} \times \text{Sample Dilution Factor}$$

(2) Calculation example:

Serum from hyperlipidemic rabbits was used for MDA detection according to the procedure table. The OD values were: standard tube 0.126, test tube 0.119, and blank tube 0.010. The control tube was replaced with a standard blank tube. The calculations are as follows:

$$\text{MDA in Hyperlipidemic Blood (nmol/mL)} = \frac{0.119-0.010}{0.126-0.010} \times 10 \times 1 = 9.40$$

Appendix IV: Methods for detecting MDA in red blood cells

(一) Detection method of MDA in trace red blood cells (for small sample sizes)

1. Sample pretreatment: (One of the following two methods can be used)

(1) Whole blood was collected on a glass slide for MDA determination:

If you need a small but precious blood sample, such as a newborn's finger prick blood or mouse tail blood, you can place 1-2 drops of heparin on a glass slide, spread it evenly, and bake it at a temperature below 60ºC or let it air dry. Place the small amount of blood sample onto the heparinized slide, use a micropipette to collect the required volume, and then prepare a 1:99 hemolyzed solution (i.e., whole blood: distilled water = 1:99).

(2) Preparation of red blood cells and hemolysate collected from glass micro-blood collection tubes:

① Use a 20μL glass micro-volume blood collection tube to collect approximately 20μL of heparin-anticoagulated whole blood from a glass slide. Place the glass capillary blood collection tube horizontally, quickly remove the bulb, and heat the longer end of the tube to one-third of the flame of an alcohol lamp until it glows red-hot and the tube end closes into a spherical shape. Measure and record the length of the blood column (a cm) using a ruler. Roll the blood collection tube up with paper, then place it end-down into a centrifuge tube. Centrifuge at 1500 rpm for 5-10 minutes. After centrifuging, use a small grinding wheel to cut and break the tube at the boundary between serum and red blood cells (or cut it directly with scissors). Invert the open end containing red blood cells into a centrifuge tube containing 1mL of distilled water, and centrifuge again at 1500 rpm for 5 minutes. At this point, the red blood cells in the capillary tube will precipitate at the bottom of the centrifuge tube. Remove the capillary tube from the centrifuge tube with tweezers and discard it. Place the centrifuge tube on a mixer and vortex to mix and prepare the hemolyzed blood.

Calculation of Blood Smear Cell Count

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$$\text{Cell Count} = \frac{\text{Total Volume of Diluent}}{\text{Blood Sample Volume}} \times 20 \times \text{Blood Smear Growth Length}$$

2. Operating steps:

① **Method 1:** (Standard operating procedure)

	Blank well	Standard well	Determination well	Control well
10 nmol/mL standard		0.1		
Anhydrous ethanol (mL)	0.1			
1:x hemolysin (mL)			0.1	0.1
Reagent 1 (mL)	0.1	0.1	0.1	0.1
Mix well (shake the centrifuge tube rack a few times).				
Reagent 2 application solution (mL)	3	3	3	3
Reagent 3 solution (mL)	1	1	1	
50% glacial acetic acid (mL)				1
Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95 ℃ water bath for 40 minutes, remove and cool in cold water, then centrifuge at 3500–4000 rpm for 10 minutes (centrifugation time should be extended below 3000 rpm to ensure complete precipitation). Take the supernatant at 532 nm (1 cm optical path), zero the plate with distilled water, and measure the OD value of each tube (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .				

Note 1: Generally, only 1 to 2 standard tubes and blank tubes need to be tested per batch. If the

sample does not have special color, turbidity or other factors , the control tube can be omitted and a blank tube can be used instead of the control tube.

Note 2: When aspirating the supernatant for colorimetric analysis , do not aspirate the precipitate at the bottom. If solid matter remains in the supernatant after centrifugation, increase the centrifugation speed or add 0.1 mL of chloroform, vortex for 60 seconds, centrifuge again, and then aspirate the supernatant.

Note 3: When reading the microplate reader, simply add 200 μL of supernatant to the 96-well plate and take the reading at 532 nm .

Standard operating procedure for standard tube OD: Follow the operating table. The OD of the standard tube minus the OD of the blank tube is 0.065 to 0.070 (approximately 0.045 when measuring 200 μL with an ELISA reader ; this value will increase as the sample volume of the standard increases).

Note 4 : If the OD of the test sample is found to be too low, the water bath time can be extended from 40 minutes to 80 minutes. However, the MDA test in the same project must be extended to 80 minutes to avoid batch-to-batch differences.

② **Method Two: (Micro-method)** (The OD values measured using this method are higher than those obtained using the standard operating method, but this does not affect the final result.)

First, dilute the prepared MDA reagent No. 3 according to the standard operating procedure with 50% glacial acetic acid at a ratio of 2:1. For example, dilute the prepared MDA… Add 50 mL of 50% glacial acetic acid to 100 mL of reagent #3 and mix well. Alternatively, prepare the exact amount needed for the following tests. (Note: The 50% glacial acetic acid is prepared by mixing 50 mL of distilled water with 50 mL of glacial acetic acid.)

	Blank well	Standard well	Determination well	Control well
10 nmol/mL standard (mL)		0.1		
Anhydrous ethanol (mL)	0.1			
Test sample (mL)			0.1	0.1
Reagent 1 (mL)	0.1	0.1	0.1	0.1
Mix well (shake the centrifuge tube rack a few times).				
Reagent 2 (mL)	1.5	1.5	1.5	1.5
Reagent 3 (mL)	1.5	1.5	1.5	
50% glacial acetic acid (mL)				1.5
Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95 °C water bath for 40 minutes, remove and cool in cold water, then centrifuge at 3500–4000 rpm for 10 minutes. Take the supernatant at 532 nm (1 cm optical path length), zero the plate with distilled water, and measure the OD value of each tube (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .				

Note 1: Generally, only 1 to 2 standard tubes and blank tubes need to be tested per batch. If the sample does not have special color or turbidity , the control tube can be omitted and a blank tube can be used instead.

Note 2: When aspirating the supernatant for colorimetric analysis , do not aspirate to the bottom precipitate. When reading the value using an ELISA reader, only 200-300 μL of supernatant needs to be aspirated into the 96-well plate, and the value should be read at 532 nm .

Note 3 : If the OD of the test sample is found to be too low, the water bath time can be extended from 40 minutes to 80 minutes. However, the MDA test in the same project must be extended to 80 minutes to avoid batch-to-batch differences.

The reference OD for the standard tube in this method is as follows: When the sample volume of the standard is 0.1 mL, the OD of the standard tube minus the OD of the blank tube, as measured by the spectrophotometer, is 0.103–0.112. When the sample volume of the standard is 0.2 mL, the OD of the standard tube minus the OD of the blank tube, as measured by the spectrophotometer, is

0.206–0.224.

3. Take a sample of hemolyzed blood at a certain concentration for hemoglobin determination:

Hb determination: Take 1 mL of 100-fold concentrated Hb test solution (**related reagents are available from our company**), add water to 100 mL to prepare the application test solution. Take 20 μL of 1:x hemolysin and add 5 mL of the diluted Hb application test solution, mix thoroughly, let stand for 5 minutes, wavelength 540 nm, optical path 1 cm, zero with distilled water, and measure colorimetrically. Record the OD value of each tube. **Hb calculation:** Multiply the OD value by 367.7 to get the Hb g/L. If the blood sample volume is very small, the sample volume and reagent volume can be reduced by half or proportionally.

4. Calculation and examples of MDA in red blood cells:

① **Calculation formula:**

$$\text{MDA (nmol/mgHb)} = \frac{\text{Determination OD} - \text{Control OD}}{\text{Standard OD} - \text{Blank OD}} \times \text{Standard Concentration (10nmol/mL)} \div \text{Blood Hemoglobin Concentration (mgHb/mL)}$$

* nmol/mgHb is nanomoles/mg of hemoglobin.

② Calculation **example:**

Example 1: 0.1 mL of a 1:99 hemolysed blood sample was analyzed for MDA using Method 1. The OD of the test tube was 0.064, the OD of the standard tube was 0.077, the OD of the standard blank tube was 0.009, and the OD of the control tube was 0.011. The control tube could also be replaced by a standard blank tube with an OD of 0.009. The Hb content in the 1:99 hemolysed blood sample was 11.08 gHb/L, or 11.08 mgHb/mL. The calculated result is:

$$\text{MDA in Blood (nmol/mgHb)} = \frac{0.064 - 0.011}{0.077 - 0.009} \times 10 \div 1.108 = 0.703$$

Example 2: 0.1 mL of a person's 1:99 hemolyzed blood was analyzed using the micro-method (Method 2). The OD of the test tube was 0.084, the OD of the standard tube was 0.100, the OD of the standard blank tube was 0.015, and the OD of the control tube was 0.017. The control tube could also be replaced by a standard blank tube with an OD of 0.015. The Hb content in the 1:99 hemolyzed blood was 11.08 gHb/L, or 11.08 mgHb/mL. The calculated result is:

$$\text{MDA in Blood (nmol/mgHb)} = \frac{0.084 - 0.017}{0.100 - 0.015} \times 10 \div 1.108 = 0.711$$

Appendix V: Method for detecting MDA in red blood cells in whole blood (for large sample sizes)

1. Sample pretreatment:

① **Blood cell preparation** : Take 0.15 mL of heparin-anticoagulated whole blood, add 2–3 mL of physiological saline, and centrifuge at 2500–3000 rpm for 5–10 minutes (mouse red blood cells are

prone to rupture, so it is best to centrifuge at 1000–1500 rpm for 5–10 minutes). Discard the supernatant and keep the precipitated red blood cells. The supernatant must be aspirated and discarded as completely as possible to avoid affecting the results.

② **Preparation of blood lysate** : Take the precipitated red blood cells after centrifugation and add 0.6 mL of distilled water (4 times the volume of the whole blood) to the precipitate. Mix in a vortex mixer for 1 minute to fully dissolve the cells.

③ **Extraction: Add 0.3 mL of anhydrous ethanol (twice the volume of whole blood)** to the above-mentioned lysed blood (95% ethanol can also be used instead) and mix thoroughly on a vortex mixer for 30 seconds.

④ **Protein precipitation : Add 0.3 mL of** chloroform (twice the volume of whole blood) and vortex for 1 minute. Then centrifuge at 3000–3500 rpm for 5–10 minutes . The liquid will separate into three layers: the upper layer is the MDA extract, the middle layer is the hemoglobin precipitate, and the lower layer is chloroform. Collect the supernatant and record the volume, approximately 0.9 mL.

2. Operating steps:

① **Preparation of 1 nmol/mL standard working solution:** Dilute the 10 nmol/mL standard 10 times, that is, take 1 mL of 10 nmol/mL standard and add 9 mL of anhydrous ethanol to prepare a 1 nmol/mL standard working solution.

② **Testing Procedure:** (If the OD of the test sample is found to be too low, the water bath time can be extended from 40 minutes to 80 minutes. However, the MDA testing in the same project must be extended to 80 minutes to avoid batch-to-batch differences.)

	Blank well	Standard well	Determination well
1 nmol/mL standard (mL)		0.8	
Anhydrous ethanol (mL)	0.8		
Extraction solution (mL)			0.8
Reagent 2 (mL)	1.0	1.0	1.0
Reagent 3 (mL)	1.0	1.0	1.0
Cap the centrifuge tubes, poke a small hole in the cap with a needle, vortex to mix, incubate in a 95 ℃ water bath for 40 minutes, remove and cool with cold water, and measure the OD value A of each tube at 532 nm with a 1 cm optical path length using distilled water (or add 200 μL of supernatant from each tube to a 96-well plate and read the value at 532 nm using a microplate reader) .			

3. Calculation and example of MDA in whole blood red blood cells:

① **Calculation formula:**

$$\text{MDA (nmol/mL)} = \frac{A_{\text{Determination}} - A_{\text{Control}}}{A_{\text{Standard}} - A_{\text{Blank}}} \times \text{Standard Concentration (10nmol/mL)} \times \frac{\text{Pre-treatment Volume (0.15mL)}}{\text{Sample Volume (0.15mL)}}$$

② **Calculation example:**

Example 1: Take 0.15 mL of whole blood from a person **, wash with physiological saline, centrifuge, discard the supernatant, add 0.6 mL of distilled water** to the precipitated red blood cells , mix for 1 minute, add **0.3** mL of anhydrous ethanol, mix for 30 seconds, then add **0.3** mL of chloroform, mix for 1 minute, centrifuge at 3500 rpm for 8 minutes, take 0.8 mL of the upper extract **for** testing, the OD of the standard tube is **0.112** , the OD of the test tube is **0.038** , and the OD of the blank tube is 0.012 **.** The result is:

$$\text{MDA in Whole Blood (nmol/mL)} = \frac{0.138-0.012}{0.082-0.012} \times 1 \times 1.05 \div 0.15 = 1.82\text{nmol/mL}$$

Appendix VI: Reference Values

The hemolytic activity of human red blood cells at a ratio of 1:99 was 6.38 ± 0.072 nmol/mL.
Human serum (plasma) 4.06±0.6 nmol/mprot,
10% rat kidney homogenate contained 2.70 ± 0.46 nmol/mg prot.
10% rat lung homogenate: 1.65 ± 0.44 nmol/mg pL.

10% rat brain homogenate contained 4.72 ± 1.44 nmol/mg rot.
10% rat myocardial homogenate contained 1.63 ± 0.36 nmol/mg prot
Mouse serum (plasma) level: 5.30 ± 0.73 nmol/mL.
10% mouse brain homogenate contained 16.3 ± 5.04 nmol/mg prot.
5% mouse myocardial homogenate contained 14.64 ± 2.68 nmol/mg prot.
10% mouse muscle homogenate contained 51.59 ± 11.08 nmol/mg prot.
10% mouse liver homogenate: 8.26 ± 2.75 nmol/mg prot.

(The above results are for reference only. Each laboratory can establish its own reference values. There are significant differences between different species of test samples. Please try to conduct a pre-test before the formal experiment.)

Appendix VII: Points to Note

1. Centrifuge tubes must be clean , especially when measuring trace samples.
2. Mix reagents thoroughly when preparing them. Discard the reagent in the first tube during the test. Add samples or reagents vertically, not onto the tube wall. Mix thoroughly before placing in a 95℃ water bath.
3. The water bath time and temperature should be kept constant. If a water bath pot is unavailable, an aluminum pot, aluminum box, or aluminum basin can be used to boil the water with the lid off.
4. Centrifugation sedimentation must be thorough, otherwise it will affect OD and cause unstable results. In this case, the centrifugation speed (above 3000 rpm) or the centrifugation time can be increased to ensure complete sedimentation.
5. If the test solution appears misty in winter, gently place it in a water bath to warm it slightly. Once the solution dissolves and becomes transparent, use a pipette to transfer it into a cuvette. If it remains misty, hyperlipidemia should be considered.
6. Sample Size: If your sample size is large, the sample size can be doubled. The amounts of anhydrous ethanol, standard, and reagent one should all be doubled , while the amounts of other reagents remain unchanged. If your sample is a blood sample from an anemic patient, the sample size should also be doubled, as should the amounts of anhydrous ethanol, standard, and reagent one , while the amounts of other reagents remain unchanged .
7. When washing red blood cells, the supernatant after centrifugation should be removed as completely as possible to ensure accurate extraction volume.
8. When using a 95℃ water bath, it is best to use centrifuge tubes with lids to prevent the reaction solution from evaporating. If centrifuge tubes with lids are not available, you can use refrigerator plastic wrap to cover them, secure them with rubber bands, and poke a small hole in the plastic wrap with a needle to replace the lid.

Appendix VIII: Advantages of the Kit

1. All reagents in this kit have no irritating odor and are not toxic to operators.
2. Fast and accurate, easy to operate, capable of testing more than 100 samples per hour.
3. High sensitivity; only 0.1 mL or less of serum or plasma sample is needed.
4. It has good reproducibility, with a coefficient of variation (CV) of 1.5%, meaning that the results of multiple tests on the same sample are very similar.
5. The color is stable, and the OD remains unchanged within 24 hours after color development.
6. The reagent is stable and has a shelf life of more than one year.
7. Serum samples stored at 4℃ will show unchanged test results for 3-5 days. They can be stored at -20℃ or below for 3 to 6 months.
8. It has a wide testing range, and can test serum (plasma), various tissue homogenates, cultured cells, etc.
9. The main raw materials are all imported, but the prices are reasonable.

10. No expensive or special instruments are required. A constant temperature water bath or aluminum pot/basin with the lid off for boiling is sufficient, as well as any model of 721, 722, 751, or 752 spectrophotometer.
11. Unaffected by external factors such as temperature. It is currently the most stable MDA testing method in China.