

### General Information

Malondialdehyde (MDA) is a highly reactive compound. Measuring MDA is important in various fields of research such as oxidative stress and ferroptosis. The MDA Assay Kit includes all the necessary components to measure MDA in cellular and tissue samples using the principle below (Figure 1). The kit can be used for both colorimetric and fluorometric assays, depending on the sample type. Compared with other commercially available MDA assay kits, little time is required for assays using this kit (approx. 60 min).

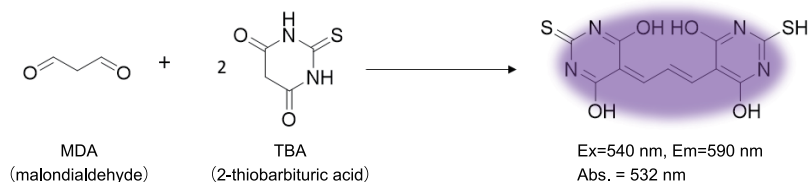


Figure 1. Principle of MDA Assay Kit

### Kit Contents

Lysis Buffer	6.5 ml×1
Dilution Buffer	10 ml×1
Standard (1 mmol/l) (Blue cap)	200 µl×1
Substrate (Red cap)	58 mg×1
Antioxidant (Green cap)	200 µl×1

### Storage Conditions

Store at -20°C

### Required Equipment and Materials

- Microplate reader (fluorometric measurement: excitation filter 540 nm, emission filter 590 nm, colorimetric: 532 nm filter)
- 96-well plate (fluorometric measurement: black; colorimetric: clear)
- 100–1000 µl, 20–200 µl, 1–10 µl micropipettes
- Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO)
- Microtubes
- Conical tubes
- Vortex mixer
- Heating block or water bath (95°C)
- Ice bath
- Homogenizer (only required if tissue samples are used)

### Precautions

- Equilibrate reagents to room temperature before use.
- Precipitate may be found in the Lysis Buffer. In this case, dissolve the precipitate by warming the solution. (recommended temperature: 40–50°C).
- Analysis of samples and standards in triplicate is recommended for accuracy.
- Prepare the MDA concentration in samples to be in the range of the standard curve (refer to Table 1).
- One kit enables the measurement of 8 standards and 24 samples in triplicate assays.

### Assay method Selection

The fluorometric method is applicable for both cell and tissue samples. For tissue samples, the colorimetric method is an alternative.

Table 1. Selection guide by sample type

Sample type	Measurement method	Measurable MDA concentration range	Sample amount*1
Cell	fluorometric	1–10 µmol/l	1–3×10 <sup>7</sup> cells
Tissue	fluorometric	1–10 µmol/l	10–30 mg
	colorimetric	10–50 µmol/l	20–50 mg

Note: For tissue samples, both the fluorometric method and the colorimetric method can be applicable.

Allow the fluorometric method to detect MDA with higher sensitivity than the colorimetric method.

\*1 Prepare an adequate volume of sample. The sample amounts listed in the Table are the minimum.

### Preparation of Solutions

#### Preparation of Substrate stock solution

Add 400 µl DMSO to the Substrate (red cap) and dissolve using sonication.

Note: The Substrate stock solution is stable for 2 months when stored at -20°C.

#### Preparation of Antioxidant PBS solution

Add an appropriate amount of PBS and Antioxidant (green cap) to a tube and mix them to prepare Antioxidant PBS solution (see Table 2 or 3).

Note: Please be aware that the antioxidant will not dissolve in PBS at this stage (resulting in a cloudy solution). The Antioxidant will be completely dissolved later in the assay procedure during the heating stage of measurement (Measurement step 3 in both fluorometric and colorimetric methods).

Note: The Antioxidant PBS solution cannot be stored and must be freshly prepared each day.

Table 2. The required amount of PBS and Antioxidant to prepare Antioxidant PBS solution for fluorometric assay

	8 samples	16 samples	24 samples
PBS	990 µl	1980 µl	2970 µl
Antioxidant	10 µl	20 µl	30 µl

Table 3. The required amount of PBS and Antioxidant to prepare Antioxidant PBS solution for colorimetric assay

	8 samples	16 samples	24 samples
PBS	4950 µl	8910 µl	14850 µl
Antioxidant	50 µl	90 µl	150 µl

## Preparation of Working solution

- (1) Add Dilution Buffer to a conical tube.
- (2) Add Substrate stock solution to the Dilution Buffer (see Table 4).

**Note:** The Working solution cannot be stored and must be freshly prepared each day.

Table 4. The required amount of Working solution by sample number

	Standard (8 serial-dilutions)	8 samples	16 samples	24 samples
Dilution Buffer	2500 $\mu$ l	2500 $\mu$ l	4800 $\mu$ l	7200 $\mu$ l
Substrate	90 $\mu$ l	90 $\mu$ l	172 $\mu$ l	260 $\mu$ l

## General Protocol

### Fluorometric Measurement

#### 1. Preparation of MDA standard solutions

- (1) Mix 10  $\mu$ l Standard (blue cap) and 990  $\mu$ l double-distilled (dd) H<sub>2</sub>O in a microtube.
- (2) Serially dilute the solution with dd H<sub>2</sub>O to prepare standards as shown in Figure 2.
- (3) Transfer 100  $\mu$ l of the 10  $\mu$ mol/l standard to a fresh tube (Figure 2).

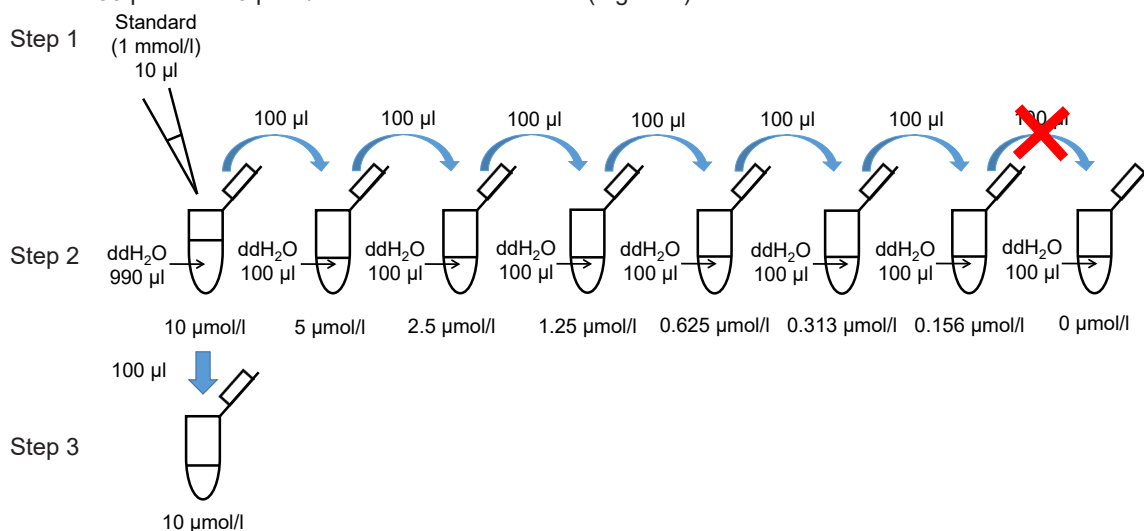


Figure 2. Preparation of MDA standard solutions

#### 2. Sample preparation

##### — Cell samples —

- (1) Prepare a cell suspension ( $1-3 \times 10^7$  cells) in a 1.5 ml microtube.

**Note:** The suitable cell number may differ depending on the cell line and treatment conditions.

- (2) Centrifuge the cells at  $300 \times g$  for 5 min and remove the supernatant.
- (3) Add 1 ml PBS and resuspend the cell pellet by pipetting.
- (4) Centrifuge the cells at  $300 \times g$  for 5 min and remove the supernatant.
- (5) Add 100  $\mu$ l Antioxidant PBS solution and resuspend the cell pellet by pipetting.

##### — Tissue samples —

- (1) Place the tissue (10–30 mg) in a 1.5 ml microtube.

**Note:** Perfuse the tissue sample before the assay as blood components may affect the measurement.

**Note:** Optimize the amount of samples for each experiment (see Table 1).

- (2) Add 300  $\mu$ l Antioxidant PBS solution to the tissue sample.
- (3) Homogenize the sample on ice.  
**Note:** Make the tissue sample as homogeneous as possible.
- (4) Transfer the homogenized sample to a 1.5 ml microtube and centrifuge at  $10000 \times g$  for 5 min.
- (5) Transfer 100  $\mu$ l of the supernatant to a fresh 1.5 ml microtube

#### 3. Measurement

- (1) Add 100  $\mu$ l Lysis Buffer to the tube (including a sample or MDA standard solution) and mix using a vortex mixer. After mixed, incubate the tube for 5 min at room temperature.

**Note:** Because of high viscosity, the solutions must be mixed by vortexing.

**Note:** Normalization of MDA concentration by protein (BCA method) is strongly recommended. In case of high viscosity, take the viscous sample into and out from a syringe 20–30 times before protein quantification.

- (2) Add 250  $\mu$ l Working solution to the tube and mix using a vortex mixer.
- (3) Incubate the tube for 15 min at 95°C.
- (4) Cool the tube for 5 min in an ice bath.
- (5) Centrifuge the tube at  $10000 \times g$  for 10 min, and transfer 100  $\mu$ l of supernatant to each well of a 96-well black plate.

**Note:** If insoluble material is observed even after the centrifugation, filter (0.22  $\mu$ m) the supernatant.

- (6) Measure the fluorescence using a microplate reader (recommended filter settings: Ex: 540 nm, Em: 590 nm).
- (7) Calculate the MDA concentration using a calibration curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µmol/l MDA			Sample 1			Sample 9			Sample 17		
B	5 µmol/l MDA			Sample 2			Sample 10			Sample 18		
C	2.5 µmol/l MDA			Sample 3			Sample 11			Sample 19		
D	1.25 µmol/l MDA			Sample 4			Sample 12			Sample 20		
E	0.625 µmol/l MDA			Sample 5			Sample 13			Sample 21		
F	0.313 µmol/l MDA			Sample 6			Sample 14			Sample 22		
G	0.156 µmol/l MDA			Sample 7			Sample 15			Sample 23		
H	0 µmol/l MDA			Sample 8			Sample 16			Sample 24		

Figure 3. Example of assay plate format ( $n = 3$ )

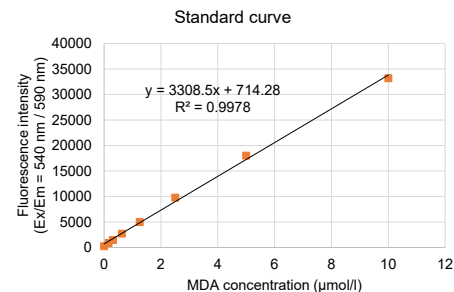


Figure 4. Typical calibration curve generated with MDA standards

## Colorimetric Measurement

### 1. Preparation of MDA standard solutions

- Mix 50 µl Standard and 950 µl double-distilled (dd) H<sub>2</sub>O in a microtube.
- Serially dilute the solution with dd H<sub>2</sub>O to prepare standards as shown in Figure 5.
- Transfer 200 µl of the 50 µmol/l standard to a fresh tube (Figure 5).

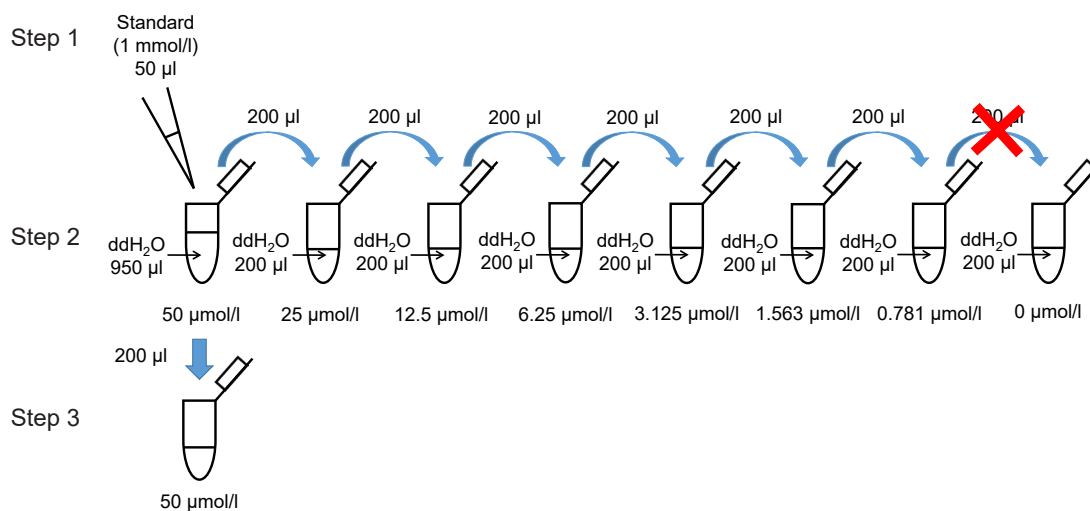


Figure 5. Preparation of MDA standard solutions

### 2. sample preparation

- Place tissue (20–50 mg) in a 1.5 ml microtube.
  - Note: Perfuse the tissue sample before the assay as blood components may affect the measurement.
  - Note: Optimize the sample amount for each experiment (see Table 1).
- Add 500 µl Antioxidant PBS solution to the tube.
- Homogenize the sample on ice.
- Transfer the solution prepared in step (3) to a 1.5 ml microtube and centrifuge at 10000 × *g* for 5 min.
- Transfer 200 µl of the supernatant to a fresh 1.5 ml microtube.

### 3. Measurement

- Add 200 µl Lysis Buffer to the tube (including a sample or MDA standard solution) and mix using a vortex mixer. After mixed, incubate the tube for 5 min at room temperature.
  - Note: Because of high viscosity, the solutions must be mixed by vortexing.
  - Note: Normalization of MDA concentration by protein (BCA method) is strongly recommended. In case of high viscosity, take the viscous sample into and out from a syringe 20–30 times before protein quantification.
- Add 300 µl Working solution to the tube and mix using a vortex mixer.
- Incubate the tube for 15 min at 95 °C.
- Cool the tube for 5 min in an ice bath.
- Centrifuge the tube at 10000 × *g* for 10 min, and transfer 200 µl of supernatant to each well of a 96-well clear plate.
  - Note: If insoluble material is observed even after the centrifugation, filter (0.22 µm) the supernatant.
- Measure the absorbance at 532 nm using a microplate reader.
- Calculate the MDA concentration using a calibration curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50 µmol/l MDA			Sample 1			Sample 9			Sample 17		
B	25 µmol/l MDA			Sample 2			Sample 10			Sample 18		
C	12.5 µmol/l MDA			Sample 3			Sample 11			Sample 19		
D	6.25 µmol/l MDA			Sample 4			Sample 12			Sample 20		
E	3.125 µmol/l MDA			Sample 5			Sample 13			Sample 21		
F	1.563 µmol/l MDA			Sample 6			Sample 14			Sample 22		
G	0.781 µmol/l MDA			Sample 7			Sample 15			Sample 23		
H	0 µmol/l MDA			Sample 8			Sample 16			Sample 24		

Figure 6. Example of assay plate format ( $n = 3$ )

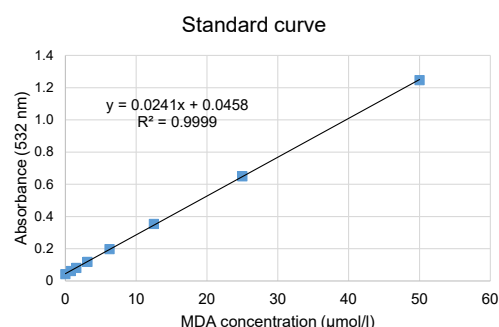


Figure 7. Typical calibration curve generated with MDA standards

### Change in intracellular MDA concentration in erastin -treated HepG2 cells

- (1) HepG2 cells were seeded in a 15 cm dish [ $1 \times 10^7$  cell/dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin] and cultured overnight in an incubator (37°C, 5% CO<sub>2</sub>).
- (2) The medium was removed, and erastin solution (100 µmol/l in DMEM) was added to the dish.
- (3) The cells were cultured for 3 h in an incubator (37°C, 5% CO<sub>2</sub>).
- (4) The medium was removed, and the cells were washed once with 10 ml PBS.
- (5) The cells were trypsinized and collected in a conical tube.
- (6) The erastin-treated HepG2 cells were transferred to a 1.5 ml tube and the cell number was adjusted to  $1 \times 10^7$  cells/tube.
- (7) Control cells were transferred to a separate 1.5 ml tube ( $1 \times 10^7$  cells/tube).
- (8) The supernatants were removed after centrifugation at  $300 \times g$  for 5 min.
- (9) PBS (1 ml) was added to each tube, and the supernatants were removed after centrifugation at  $300 \times g$  for 5 min.
- (10) Antioxidant PBS solution (100 µl) was added to each tube, and the pellet was resuspended by pipetting.
- (11) Lysis Buffer (100 µl) was added to each tube, and the solution was mixed using a vortex mixer.
- (12) The tubes were incubated at room temperature for 5 min.
- (13) Working solution (250 µl) was added to each tube, and the solution was mixed using a vortex mixer.
- (14) The tubes were incubated at 95°C for 15 min.
- (13) The tubes were cooled on ice for 5 min.
- (14) The supernatant (100 µl) was transferred to each well of a 96-well black plate after centrifugation at  $10000 \times g$  for 10 min.
- (15) Fluorescence was measured using a microplate reader (Ex: 540 nm, Em: 590 nm), and the concentration of MDA in the samples was calculated from the calibration curve.

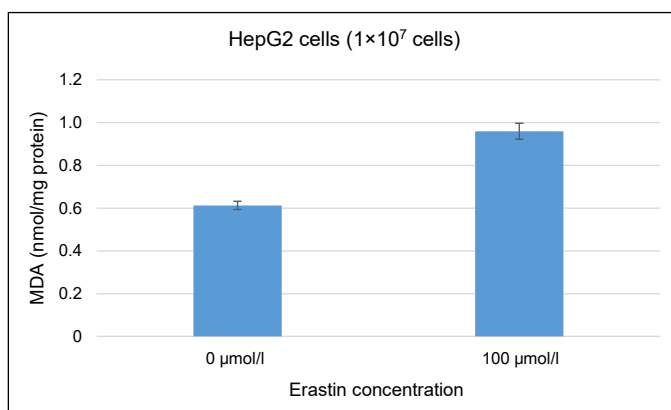


Figure 8. Determination of intracellular MDA concentration in erastin-treated and non-treated HepG2 cells

### Measurement of MDA concentration in KK-Ay mouse liver

- (1) Antioxidant PBS solution (500 µl) was added to a 1.5 ml microtube containing the tissue sample (30 mg).
- (2) The tissue sample was homogenized using a Dounce homogenizer.
- (3) The solution prepared in step (2) was transferred to a 1.5 ml microtube.
- (4) The supernatant (200 µl) was transferred to a fresh 1.5 ml microtube after centrifugation at  $10000 \times g$  for 5 min.
- (5) Lysis Buffer (200 µl) was added to the tube, and the solution was mixed using a vortex mixer.
- (6) The tube was incubated at room temperature for 5 min.
- (7) Working solution (300 µl) was added to the tube, and the solution was mixed using a vortex mixer.
- (8) The tube was incubated at 95°C for 15 min.
- (9) The tube was cooled on ice for 5 min.
- (10) The supernatant (200 µl) was transferred to each well of a 96-well clear plate after centrifugation at  $10000 \times g$  for 10 min.
- (11) Absorbance at 532 nm was measured using a microplate reader, and the concentration of MDA in the samples was calculated from the calibration curve.

Table 5. MDA concentration in KK-Ay mouse liver

	MDA concentration (µmol/l)
KK-Ay mouse liver (30 mg)	21.15

For research use only. Not for use in diagnostic procedures.  
If you need more information, please contact Dojindo technical service.