ab118970 Lipid Peroxidation (MDA) Assay kit (Colorimetric/Fluorometric)

For the measurement of Lipid Peroxidation in plasma, cell culture and tissue extracts.
For research use only – not intended for diagnostic use.

For overview, typical data and additional information please visit: http://www.abcam.com/ab118970 (use http://www.abcam.cn/ ab118970 for China, or http://www.abcam.co.jp/ab118970 for Japan)

Background:

Lipid Peroxidation (MDA) Assay kit (Colorimetric/Fluorometric) (ab118970) is a chemical based sensitive method for measuring MDA in plasma, cell culture extracts, tissue extracts, and urine (UTI).

MDA in the sample is reacted with Thiobarbituric Acid (TBA) to generate the MDA-TBA adduct which can be easily quantified colorimetrically (OD 532 nm) or fluorometrically (Ex/Em = 532/553 nm). This assay detects MDA levels as low as 1 nmol/well colorimetrically and 0.1 nmol/well fluorometrically

Assay Summary:

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

Sample preparation (in tubes)

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Standard curve preparation (in tubes)

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Add Developer reaction mix and incubate 60min at 95°C. Cool to room temperature using an ice bath for 10 minutes

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Move 200 µL of each standard and sample to plate

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Measure optical density (OD532 nm) or fluorescence (Ex/Em = 532/553 nm)

QUICK ASSAY PROCEDURE

- Preheat heat block to 95°C
- Thaw all components. Mix Developer VII with 7.5 mL glacial acetic acid and adjust final volume to 25 mL
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare MDA-TBA adduct by adding 600 µL of Developer Reaction Mix.
- Heat at 95 °C for 1 hour, cool to RT by putting on ice for 10 minutes
- Pipette standard (200 µL) and samples (200 µL) into 96-well plate.
- Measure plate at OD 532nm for colorimetric assay or Ex/Em= 532/553 nm for fluorometric assay.

Precautions & Limitations:

Please read these instructions carefully prior to beginning the assay.
All kit components have been formulated and quality control tested to function successfully as a kit.

- Modifications to the kit components or procedures may result in loss of performance.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

Storage and Stability:

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Reconstituted Developer Mix R is stable for 1 week. Do not use kit or components if they have exceeded the expiry date.

Materials Supplied:

Item	Quantity	Storage Temperature (on receipt)	Storage temperature (reconstituted)
MDA Lysis Buffer	25 mL	-20°C	-20°C
Phosphotungstic Acid Solution	12.5 mL	-20°C	-20°C
BHT Stock	1 mL	+4°C	-20°C
Developer Mix R	4 x 250 mg	Ambient	+4°C
MDA Standard	100 µL	-20°C	-20°C

PLEASE NOTE: Developer Mix R was previously labeled as Developer VII and TBA Solution. The composition has not changed.

Materials Required, Not Supplied:

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at OD 532 nm (colorimetric) or fluorescence at Ex/Em = 532/553 nm (fluorometric)
- 96 well clear plate with clear flat bottom (colorimetric assay) or 96 well black plate with flat bottom (fluorometric assay)
- Glacial Acetic Acid
- 42 mM H_2SO_4 – for plasma and serum sample preparation
- Sonicator (Optional) – to help solubilize Developer Mix R
- n-Butanol (Optional) – to increase assay sensitivity
- 5 M NaCl (Optional) – to increase assay sensitivity
- 2 N perchloric acid (Optional) – for alternative cell/tissue preparation protocol
- Dounce homogenizer (if using tissues or cells)
- Sonicator (if using cells)
- 1x PBS pH 7.4 (
- MilliQ water or other type of double distilled water (ddH2O)

Reagent Preparation:

- Briefly centrifuge small vials at low speed prior to opening.
- Equilibrate reagents to room temperature before use.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.

MDA Lysis Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Aliquot buffer so that you have enough to perform the desired number of assays.

Phosphotungstic Acid Solution: Ready to use as supplied. Equilibrate to room temperature before use.

BHT Stock: Ready to use as supplied. Equilibrate to room temperature before use. Aliquot solution so that you have enough to perform the desired number of assays.

Developer Mix R: Reconstitute one vial of Developer Mix R (250 mg) in 7.5 mL Glacial Acetic Acid (use only Glacial acetic acid, as regular acetic acid affects Developer Mix R stability due to its high-water content). Transfer slurry to another tube and then adjust the final volume to 25 mL with ddH_2O, referred to as Developer Reaction Mix. Mix well to dissolve. Sonicate in a RT water bath if required. Reconstituted solution is stable for 1 week at +4°C or -20°C. We do not recommend aliquoting as it may result in inconsistencies.
*** Note:** In case of precipitate formation, sonicate in a water bath (RT). Alternatively, Developer Mix R can also be dissolved in 15 mL of 50% glacial acetic acid in water, then making up volume to 25 mL with ddH2O (use molecular grade deionized distilled water). Mix well. If there are still precipitates, use 600 µL as directed (Generation of MDA- Developer Mix R adduct, step 1.1), any particle in suspension will dissolve at 95°C when incubated for 60 minutes.

MDA Standard: Ready to use as supplied. Aliquot standard so that you have enough to perform the desired number of assays.

Sample Preparation:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

1. We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Cells (adherent or suspension) samples:

1. Harvest the number of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).
2. Wash cells in cold PBS.
3. Resuspend cells in 300 µL of MDA Lysis Buffer with 3 µL BHT Stock. BHT stops further sample peroxidation while processing.
4. Homogenize cells in 303 µL Lysis Solution (Buffer + BHT) using a Dounce homogenizer (10-50 passes) on ice.
5. The DNA released from the cells could make the lysate viscous. Sonicate the lysate before centrifugation to shear the DNA and reduce the viscosity of the solution.
6. The cells can be observed under microscope for efficient lysis, a shiny ring around nuclei indicate cells are still intact (perform 30-50 additional passes). If 70 – 80% of the nuclei do not have the shiny ring, proceed to the next step.
7. Centrifuge at 13,000 x *g* for 10 minutes to remove insoluble material)
8. Collect supernatant and transfer to a clean tube.
9. Keep on ice.

Tissue Samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
2. Wash tissue in cold PBS.
3. Add tissue to 300 µL of MDA Lysis Buffer with 3 µL BHT Stock. BHT stops further sample peroxidation while processing.
4. Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
5. Centrifuge at 13,000 x *g* for 10 minutes to remove insoluble material.
6. Collect supernatant and transfer to a clean tube.
7. Keep on ice.

***Note:** For samples containing high amount of protein, we recommend using the following modified protocol:

1. Precipitate protein by homogenizing 1-10 mg tissue samples in 150 µL ddH2O + 3 µL BHT. BHT is normally miscible with water however in case of precipitate formation tissue homogenization helps in dissolution.
2. Add 1 volume of 2N perchloric acid, vortex and then centrifuge to remove precipitated protein.
3. Collect 200 µL of supernatant from tissue sample and transfer to a clean microcentrifuge tube.

Plasma Samples:

1. Gently mix 20 µL plasma or serum with 500 µL of 42 mM H_2SO_4 in a microcentrifuge tube. Plasma can be collected using any anticoagulant.
2. Add 125 µL of Phosphotungstic Acid Solution and mix by vortexing. This acid will preferentially precipitate lipids and the resulting pellet should have low contamination with proteins.
3. Incubate at room temperature for 5 minutes.
4. Centrifuge at 13,000 x *g* for 3 minutes.
5. Collect the pellet and resuspend on ice with 100 µL ddH_2O (with 2 µL BHT Stock).If the pellet doesn't dissolve and sonicate in a water bath**.** Adjust final volume to 200 µL ddH_2O
   ***Note:** If lipids form a suspension as these are not miscible with water, just proceed with the protocol. The Developer Mix R will react with the MDA in these lipid particles. Sonicate as needed.
6. Adjust the final volume to 200 µL with ddH2O.
   ***Note:** Mild plasma hemolysis shouldn't affect the assay. If hemolysis is observed we recommend fluorometric method.
   ***Note:** Urine samples can also be used directly with this kit. The samples should be assayed immediately after collection for best results, however samples stored at -70°C can be used.

Standard Preparation:

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.
- Each dilution has enough standard to set up triplicate readings (3 x 200 µL).
- If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.

Prepare serial dilution of MDA Standard as follows:

1. 0.1 M dilution: Add 10 µL of 4.17 M MDA Standard to 407 µL ddH_2O. Gently pipette up and down a few times to ensure all standard is removed from tip. Mix well by inversion.
2. 2 mM dilution: Transfer 10 µL of 0.1 M dilution to 490 µL ddH_2O. Gently pipette up and down and then mix well by inversion. **Use this to prepare standard curve for colorimetric assay.**
3. 0.2 mM dilution: Transfer 10 µL of 2 mM dilution to 490 µL ddH_2O. Gently pipette up and down and then mix well by inversion. **Use this to prepare standard curve for fluorometric assay.**

For colorimetric assay: Using 2 mM MDA standard, prepare standard curve dilution as described in the table below in microcentrifuge tubes:

For fluorometric assay: Using 0.2 mM MDA Standard, prepare standard curve dilution as described in the table below in microcentrifuge tubes:

Standard #	Volume of 2 mM or 0.2 mM MDA Standard (µL)*	Volume ddH_2O (µL)	Final volume standard in microfuge tube (µL)	Amount of MDA-TBA adduct (nmol/well) Colorimetric Assay	Amount of MDA-TBA adduct (nmol/well) Fluorometric Assay
1	0	200	200	0	0
2	2	198	200	1	0.1
3	4	196	200	2	0.2
4	6	194	200	3	0.3
5	8	192	200	4	0.4
6	10	190	200	5	0.5

***NOTE: For colorimetric assay use 2 mM standard, for fluorometric assay use 0.2 mM standard**
***NOTE: do not set up in plate. MDA-TBA adduct will be made in microfuge tubes then an aliquot transferred to plate once cooled.**

Assay Procedure:

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.

- We recommend that you assay all standards, controls, and samples in duplicate.

1. Generation of MDA-TBA adduct:

1. Add 600 µL of Developer Mix (diluted developer VII) into each vial containing 200 µL of standard or sample (final volume 800 µL).
2. Incubate at 95°C for 60 minutes.
3. Cool to room temperature in an ice bath for 10 minutes.
   ***Note:** Occasionally samples will exhibit a turbidity which can be eliminated by filtering them through a 0.2 µm filter. Developer Mix R can also react with other compounds in samples giving other colored compounds, these however should not interfere with quantitation of the Developer Mix R-MDA adduct.
   Optional steps for enhanced sensitivity (recommended for plasma):
   *** Note:** Use this step for plasma and other samples where MDA-TBA adduct concentration is low.
   a. Add 300 µl n-butanol (not provided in the kit) to extract the MDA-TBA adduct from the 800 µl reaction mixture.
   b. If there is no separation, add 100 µl of 5 M NaCl and vortex vigorously.
   c. Centrifuge for 3 minutes at 16,000 x *g* at room temperature for better separation.
   d. Transfer the top layer (n-butanol) a new tube and evaporate by freeze-drying or by incubating sample overnight in a dry-oven at 55°C.
      *Note: A vacuum dry oven will increase the drying speed if available.
   e. Resuspend concentrated sample in 200 µL of ddH_2O.

4. Take 200 µL of the reaction mix (containing MDA-TBA adduct) and add into a 96-well microplate for analysis (the end range for the standard curve will be:
   Colorimetric: 0,1, 2, 3, 4, 5 nmol MDA-TBA adduct/well
   Fluorometric: 0, 0.1, 0.2, 0.3, 0.4, 0.5 nmol MDA-TBA adduct/well

2. Measurement:

Measure absorbance immediately on a microplate reader at OD 532 nm for colorimetric assay and RFU at Ex/Em = 532/553 nm for fluorometric assay.
Δ Note: For fluorometric assay, we recommend setting the instrument sensitivity to high with a monochromator slit width of 5 nm (no cutoff filter).

Calculations:

1. Average the duplicate reading for each standard and sample.
2. Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance (or $RFU_{corrected}$ for fluorescence assays).
3. Plot the corrected absorbance (or $RFU_{corrected}$) values for each standard as a function of the final concentration of MDA.
4. Determine the equation of the standard curve using a linear regression.
5. Apply the corrected sample absorbance (or $RFU_{corrected}$) to the standard curve to get MDA amount (nmol) in the sample wells.

$$B = \left(\frac{A_{corrected} - (y - intercept)}{Slope}\right)$$

6. Concentration of MDA in the test samples is calculated as:

$$MDA\ Concentration = \left(\frac{B}{[M\ or\ V]}\right) \times\ 4^{*}\ \times\ D = \frac{nmol}{mg} or\ \frac{nmol}{ml}$$

Where:
B = Amount of MDA in sample calculated from the standard curve (in nmoles).
M = Original tissue mass used in mg.
V = Original plasma volume used (in mL; i.e. 0.020 mL).
4* = Correction for using 200 µL of the 800 µL Reaction Mix.
D^{*} = Sample dilution factor if sample is diluted to fit within the standard curve range or not all of the sample was used in the MDA-TBA adduct step(prior to reaction well set up).

This correction factor (4) does not apply if the optional steps for increased sensitivity was followed as the entirety of the sample is loaded.
*If the amount of supernatant obtained after homogenization and centrifugation is > 200 µl correct for this in the dilution factor D. The total amount of supernatant that can be collected depends on the cell debris, insoluble material and tissue type.

For example, when using tissue extracts, if 300 µL of supernatant is recovered after homogenization and centrifugation and 200 µL is carried forward for generation of the MDA-TBA adduct, a dilution factor of 300/200, or 1.5 is required.

*** Note:** For tissue samples, concentration can also be expressed in nmoles/mg or nmoles/µg of protein, if a protein quantification assay has been performed.

Technical Hints

For additional helpful hints and tips on using our assay kits please visit:
https://www.abcam.com/en-us/support/product-support

Technical Support

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