KAMIYA BIOMEDICAL COMPANY

LPO-CC
(Lipid Peroxides) Kit

For the Quantitative Colorimetric Determination of High Levels of Lipid Peroxides in Serum, Plasma and Other Biological Samples.

Cat. No. CC-004

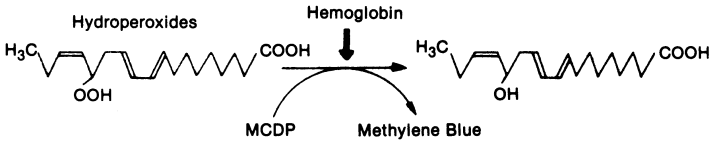
For Research Use Only. Not for Use in Diagnostic Procedures in the U.S.

PRODUCT INFORMATION

LPO-CC Kit
Cat. No. CC-004

PRODUCT
Lipid peroxides are normally present in very low levels in normal human serum and plasma (0-1.3 nmol/mL). Therefore, this assay is best used to screen for high serum or plasma LPO values or to measure LPO in other biological materials. For research use only. Not for use in diagnostic procedures in the U.S.

ADVANTAGES
1. No sample extraction required for serum or plasma.
2. Specifically quantitates lipid peroxides.
   Does not measure metabolites of lipid peroxides.
3. Applicable to automated chemistry analyzers.
4. Manual or automated procedure.



DESCRIPTION
Lipid peroxides in serum are produced by auto-oxidation of unsaturated fatty acids in the human body. The quantitative determination of lipid peroxides is believed to be useful for checking the disease state and prognosis of various liver diseases and diabetes.
Lipid peroxides are very unstable in the body and are present in very low levels. Therefore, it is necessary to directly determine lipid peroxide levels by a quick and simple assay such as the ***K-ASSAY®*** LPO-CC.

PRINCIPLE
In the presence of hemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols) and the MCDP chromogen is oxidatively cleaved to form methylene blue in an equal molar reaction. Lipid peroxides are quantitated by colorimetrically measuring the methylene blue at 675 nm.

COMPONENTS
1.	Reagent 1A: Lyophilized Enzyme Reagent:	1 x 55 mL bottle of Ascorbic Oxidase & Lipoprotein Lipase Stabilizer
2.	Reagent 1B: Buffer Solution (liquid):	1 x 55 mL bottle of Goods Buffer, 100 mM Surfactants
3.	Reagent 2A: Lyophilized Chromogen Reagent:	1 x 110 mL bottle of MCDP
4.	Reagent 2B: Buffer Solution (liquid):	1 x 110 mL bottle of Goods Buffer and Hemoglobin Surfactants
5.	Calibrator: (liquid):	1 x 10 mL bottle of 50 nmol/mL Cumene Hydroperoxide

Materials Required But Not Supplied
- Spectrophotometer: capable of accurate absorbance reading at 675 nm with appropriate cuvettes.
- Pipettes: capable of dispensing accurate volumes.
- Test Tubes: glass or plastic.
- Water Bath: capable of maintaining 30°C.

SPECIMEN COLLECTION AND PREPARATION
Serum or plasma should be separated immediately from blood samples. The assay should be run within a few hours. Samples can be stored but not for longer than 2 weeks under any storage conditions. The ***K-ASSAY®*** LPO-CC kit can be used on biological samples other than serum and plasma. However, an initial sample extraction step may be required (see below).

AUTOMATED ANALYZER APPLICATION

Suitable for two-reagent automated analyzers. Refer to the instrument manufacturer's manual for the following:

a) Use or function.
b) Installation procedures and requirements.
c) Principles of operation.
d) Performance characteristics and specifications.
e) Operation instructions.
f) Calibration procedures including materials and/or equipment to be used.
g) Operational precautions and limitations.
h) Hazards.
i) Service and maintenance.

Suggested applications are currently available for the following instruments: Cobas Fara, Mira; Olympus Reply, Boehringer Mannheim/Hitachi 704, 717, 736, 747, 911; AU-560; Technicon AXON (Toshiba 60R), Wako 30R (Toshiba 20R).

PROCEDURE

Reagent Preparation

Reagent 1 (R-1): Dissolve Reagent 1A in Reagent 1B. Incubate for 15 min. at 37°C after dissolving.
Reagent 2 (R-2): Dissolve Reagent 2A in Reagent 2B. Incubate for 15 min. at 37°C after dissolving.

Manual Assay Procedure

- ← Sample (S), Calibrator (Cal), or Saline Blank (B): 0.1 mL
↓
- ← Add Reagent 1 (Enzyme Reagent): 1.0 mL
↓
Mix, incubate at least 5 min at 30°C
↓
- ← Add Reagent 2 (Chromogen Reagent): 2.0 mL
↓
Mix, incubate at least 10 min at 30°C
↓
- ← Read absorbance at 675 nm

Es = Sample absorbance
Ecal = Absorbance of 50 nmol/mL Calibrator
Eb = Blank absorbance

Sample and reagent volumes can be changed provided that the following ratios remain constant:
Sample:Reagent = 1:30
Reagent 1:Reagent 2 = 1:2

All samples should be assayed and measured at a constant time after addition of R-2. A staggered addition of reagents can be done to keep the assay timing consistent from sample to sample.

Calibration Curve

A two-point calibration curve is made using a saline blank (0 nmol/mL) and the 50 nmol/mL Cumene Hydroperoxides Calibrator provided with the kit.

Tissue Extraction Procedures

1. Detergent Extraction (60-80% recovery expected).

- Homogenize 1 g tissue.
- Extract with 0.1% Triton X-100 and 0.05% sodium deoxycholate (desoxycholic acid) in 4 mL saline solution with shaking for 10-15 min in a cool, dark place. Triton X-100 sometimes contains peroxides so "peroxide-free" Triton X-100, freshly prepared, is preferable. Extraction time can be prolonged if the extraction tube is filled with nitrogen.
- Centrifuge to remove cellular debris.
- Dilute sample 10 fold (suggested dilution) with PBS.
- Test 0.1 mL sample using ***K-ASSAY®*** LPO-CC kit.

2. $CHCl_3$/Methanol Extraction of Phosholipids.

- Homogenize 1 g tissue in 15 mL $CHCl_3$/methanol (2:1, v/v).
- Centrifuge, mix supernatant with 3 mL 0.9% NaCl to clarify. Shake, centrifuge, remove supernatant.
- Evaporate $CHCl_3$ layer with argon or nitrogen gas or in vacuum, keeping temperature as low as possible and time as short as possible during evaporation.
- Dissolve lipid residue with isopropanol or methanol.
- Test 0.1 mL sample using ***K-ASSAY®*** LPO-CC kit.
- Ref.: Yagi, K. et al. (1986). Biochem. Internatl. **12**: 367-371.

3. Isopropanol Extraction of Phospolipids

- Combine 50 mg homogenized tissue with 2 mL isopropanol. Shake vigorously for 2 min.
- Centrifuge at 3,000 x g for 10 min, remove supernatant.
- Test 0.1 mL supernatant using **K-*ASSAY*®** LPO-CC kit.

CALCULATIONS

LPO values can be determined from a graph of the calibration curve or more accurately using the following equation:
LPO value in nmol/mL = (**Es** - **Eb**) x 50.0 / (**Ecal** - **Eb**).

AUTOMATED ASSAY PROCEDURE (Example)

Sample (S):	20 µL
↓	
• ← Add Reagent 1 (Enzyme Reagent):	80 µL
↓	
Mix, incubate at least 5 min at 30°C	
↓	
• ← Add Reagent 2 (Chromogen Reagent):	160 µL
↓	
Mix, incubate at least 10 min at 30°C	
↓	
• ← Read absorbance at 660/800 nm	

Parameters for Automated Method (Example)

Instrument :	Hitachi 717	CAL.(4) CONC.-POS.	( )-( )
Temperature:	30°C	CAL.(5) CONC.-POS.	( )-( )
TEST	(LPO)	CAL.(6) CONC.-POS.	( )-( )
ASSAY CODE	(1 POINT):(50)-(0)	SD LIMIT	(999.9)
SAMPLE VOLUME	(20)	DUPLICATE LIMIT	( )
R1 VOLUME	(80) (100) (NO)	SENSITIVITY LIMIT	(0)
R2 VOLUME	(160) (100) (NO)	ABS. LIMIT (SLOPE)	( )( )
WAVELENGTH	(800)-(660)	PROZONE LIMIT	(0)(LOWER)
CALIB. METHOD	(LINEAR)(0)	EXPECTED VALUE	( )( )
CAL.(1) CONC.-POS.	(0.0)-( )	PANIC VALUE	( )( )
CAL.(2) CONC.-POS.	(50)-( )	INSTRUMENT FACTOR	(1.00)
CAL.(3) CONC.-POS.	( )-( )		

Parameters for other automated analyzers are available.

LIMITATIONS

The linear range for this assay is between 2.0 nmol/mL to 300 nmol/mL for quantitative determination. Above this level, values plateau and are unreliable.

PERFORMANCE

- Sensitivity: When a blank is used as a sample, the absorbance should be below 0.100. When a Calibrator containing 50 nmol/mL of cumene peroxides is assayed, the absorbance should be within the range of 0.115-0.135 after subtracting the blank.
- Specificity: When lipid control samples are assayed, specificity is within ± 10%.
- Precision: The absorbance C.V. is less than 3% for 10 repeated assays.
- Assay Range: 2-300 nmol/mL for quantitative determination.
- Number of Tests: Dependent upon cuvette volume (3 mL = 55 tests, 1 mL = 165 tests, Microtiter plate = 550 tests).
- Correlation: The amount of linoleic acid hydroperoxide measured by the iodometric method and by this method gave a linear and equimolar correlation (r = 0.952).[1,3]

EXPECTED VALUES
Each laboratory should establish its own expected values using this kit.

WARNINGS AND PRECAUTIONS
- Not for internal use in humans or animals. Normal laboratory precautions should be followed while handling reagents.
- **Very small amounts of detergent residue on the test tubes will interfere with the assay, resulting in false high values.**
- Washed test tubes and cuvettes should be filled with distilled water and left for 30 min before using. With automated analyzers, careful washing of the reaction line is required.
- Substances which raise the blank reading may be liberated from some amber-colored test tubes.
- **All reaction tubes should be shielded from light during incubation and measurements.**
- Serum or plasma should be separated immediately from blood samples and the assay run within a few hours. Varying the time between sampling and analysis of similar samples may cause variation in the observed level of lipid peroxides.
- Lipid peroxides are normally present in very low levels in normal human serum and plasma (0-1.3 nmol/mL). Therefore, this assay is best used to screen for high serum or plasma LPO values or to measure LPO in other biological materials.
- Do not mix or use reagents from one test kit with those from a test kit with a different lot number.
- Do not use reagents past the expiration date stated on each reagent container label.
- Do not pipette reagents by mouth. Avoid ingestion and contact with skin.

INTERFERING MATERIAL
- Normal levels of bilirubin will not interfere with the assay. However, bilirubin levels >12 mg/dL will result in false high LPO values.
- Ordinary amounts of heparin, EDTA, and sodium fluoride will not interfere with the assay.
- Avoid exposing reagents to light, dust, NaCl, and metal ions such as Cu, Fe, Mn, Co, which interfere with the assay.
- Results may be invalid for lipemic samples.

QUALITY CONTROL
A quality control program is recommended. It is recommended that control serums, both normal and abnormal, be run with each batch of samples to monitor the procedure. The values obtained for controls should fall within the manufacturers specified range.

STORAGE
Store all unopened kit components at 4°C. Unopened reagents stored at 4°C and protected from light are stable until the expiration date on the label. **Dissolved (reconstituted) reagents are stable for 3 days when stored in the dark at 4°C and protected from light by wrapping the vials in foil. If aliquoted and stored frozen in the dark at -20°C, they are stable for 2-3 months. Do not refreeze aliquoted reagents.**

REFERENCES
1. Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., and Yagi, K. (1985). Biochem. Int. 10:205-211.
2. Tateishi, T., Yoshimine, N., and Kuzuva, F. (1987). Exp. Gerantol. 22:103-111.
3. Pervaiz, S., Harriman, A., Gulliya, K.S. (1992). Free Radical Biol. Med. 12:389-396.
4. Ma, Y., Stone, W., LeClaire, I. (1994). P.S.E.B.M. 206:53-59.
5. Supnet, M., David-Cu, R., and Walther, F. (1994). Ped. Res. 36: 283-287.
6. Wang, M.-Y. and Liehr, J. (1994). J. Biol. Chem. 269: 284-291.
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K-*ASSAY* ® LPO-CC Assay vs. TBA Assay
The TBA assay measures the acid breakdown of lipid peroxides into malonaldehyde (MDA), which then reacts with TBA to produce substances that can be measured by colorimetry. MDA comes mainly from certain peroxides such as linoleic and linolenic peroxides, but not oleic, stearic, or palmitic. TBA also measures some other aldehydes at varying sensitivities. Results therefore vary from specimen to specimen depending upon the aldehydes present.

The LPO-CC assay specifically and directly measures LPO. In the presence of hemoglobin, lipid peroxides are reduced to hydroxyl derivatives (lipid alcohols) which oxidatively cleave colorless MCDP into methylene blue. Normally, the **K-*ASSAY*®** LPO-CC kit gives higher readings with greater accuracy than TBA. However, because hydroperoxides are unstable and

degrade to produce aldehyde products over time, if the sample is not fresh or stored properly and such degradation was allowed to occur, the TBA values would increase and appear larger than those obtained with the **K-*ASSAY*®** LPO-CC kit.

The length of the hydrocarbon portion of the peroxide does not correlate with its reactivity with MCDP. The structure appears to be more important. Each lipid alcohol has a different potential for oxidizing MCDP. Alcohols derived from R-OOH type peroxides (such as cumenhydroperoxide) all have relatively the same oxidation potential. Alcohols from R-OO-R type peroxides (such as benzoylperoxide) have a lower oxidation potential. Since the LPO-CC assay measures color production within a set time, these will not be measured to the same degree since they oxidize little MCDP. Hydrogen peroxide, benzoperoxide, and peracetic acid oxidize MCDP very slowly and will not be detected in the assay.

In general type I, II, and III peroxides oxidize MCDP at varying rates. Type IV peroxides do not oxidize MCDP and type V peroxides have not been tested.

FOR RESEARCH USE ONLY

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