



Lipid Hydroperoxide (LPO) Assay Kit (96 wells)

ALX-850-203-KI01

96 wells (~80 tests)

(Version 01: August 9, 2000)

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For laboratory use only. Not for human or diagnostic use.

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CONTENTS OF THE KIT

Number	Item	Quantity
1	FTS Reagent 1	1 vial
2	FTS Reagent 2	1 vial
3	Lipid Hydroperoxide Standard	1 vial
4	Extract R	1 vial
5	Triphenylphosphine	1 vial
*6	Glass 96 Well Plate	1 plate

**The Glass 96 Well Plate is only included as a component of this 96-well size (Prod. No. ALX-850-203).*

PRECAUTIONS

- Please read these instructions carefully before beginning this assay.
- For research use only. Not for human or diagnostic use.

STORAGE AND STABILITY

Store the Lipid Hydroperoxide Standard (vial #3) at -80°C. The remaining components of the kit should be stored at 0-4°C and used before the expiration date indicated on the outside of the box.

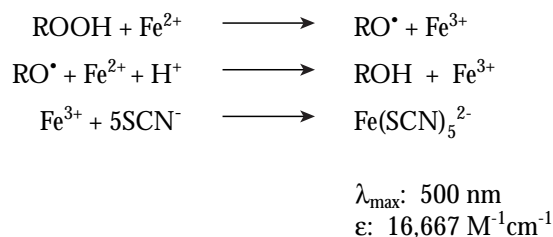
ADDITIONAL ITEMS REQUIRED

1. A spectrophotometer.
2. An adjustable pipettor.
3. A source of pure water. Glass distilled water or HPLC-grade water is acceptable.
4. Degassed chloroform and methanol (described on page 3).
5. A plate reader with a 500 nm filter (needed to use the 96 glass well plate).

ABOUT THIS ASSAY

Quantification of lipid peroxidation is essential to assess the role of oxidative injury in pathophysiological disorders.¹⁻³ Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA) and 4-hydroxy nonenal (4-HNE), the degradation products of polyunsaturated fatty acids hydroperoxides.⁴⁻⁶ Sensitive colorimetric assays have been developed to measure these aldehydes.⁴ However, these assays are non-specific and often lead to an over estimation of lipid peroxidation. There are important additional problems in using these by-products as indicators of lipid peroxidation. The by-product formation is highly inefficient and varies according to the transition metal ion content of the sample. Only hydroperoxides derived from polyunsaturated fatty acids give rise to these by-products. For example, 4-HNE is formed only from ω -6 polyunsaturated fatty acid hydroperoxides and is catalyzed by transition metal ions like ferrous.⁵ Decomposition of hydroperoxides derived from abundant cellular lipids such as cholesterol and oleic acid does not produce MDA or 4-HNE. These factors can lead to an under-estimation of lipid peroxidation. MDA is also produced in ng/ml concentrations by the platelet enzyme thromboxane synthase during whole blood clotting and platelet activation.⁷ This leads to gross over-estimation of lipid peroxidation. Estimation of lipid hydroperoxide levels range from 0.3-30 μ M depending on the method used. However, direct methods of estimation suggest that the concentration in normal human plasma is approximately 0.5 μ M.^{8,9} Given the limitations of the indirect methods, direct measurement of hydroperoxides is the obvious choice.

Alexis Biochemical Company's Lipid Hydroperoxide Assay Kit measures the hydroperoxides directly utilizing the redox reactions with ferrous ions (see Scheme 1).¹⁰ Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions are detected using thiocyanate ion as the chromogen.



Scheme 1. Reduction/Oxidation Reactions

Since this method relies on the measurement of ferric ions generated during the reaction, ferric ions present in the sample are a potential source of error. Also, many biological samples contain hydrogen peroxide which readily reacts with ferrous ions to give an over estimation of lipid hydroperoxides. These problems are easily circumvented by performing the assay in chloroform.

An easy to use, quantitative extraction method was developed to extract lipid hydroperoxides into chloroform and the extract is directly used in the assay. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a sensitive and reliable assay for lipid peroxidation.

SENSITIVITY

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.25 - 5 nmol hydroperoxide per assay tube.

PRE-ASSAY PREPARATION

Preparation of reagents

1. FTS Reagent 1: This vial contains 4.5 mM ferrous sulfate in 0.2 M hydrochloric acid. The reagent is ready to use as supplied.
2. FTS Reagent 2: This vial contains a 3% methanolic solution of ammonium thiocyanate. The reagent is ready to use as supplied.
3. Lipid Hydroperoxide Standard: This vial contains 50 μM ethanolic solution of 13-HpODE (13-hydroperoxy octadecadienoic acid). Store the standard at -80°C and keep it on ice during the experiment. The standard is ready to use as supplied.
4. Extract R: This vial contains a crystalline solid used for extraction of samples. Prepare a saturated solution of Extract R as follows: Weigh about 100 mg of the solid in to a test tube, add 15 ml methanol and vortex thoroughly for about 2 min. The methanol will become cloudy and most of the solid remains undissolved. Use the Extract R saturated methanol within 2 h. **CAUTION:** Extract R is acidic and hence direct contact should be avoided. If exposed, wash thoroughly with cold water. Excess reagent can be disposed of in sanitary sewer drains after dilution in 10 volumes of water.
5. Triphenylphosphine: This vial contains crystalline triphenylphosphine. Weigh 2.6 mg of triphenylphosphine and dissolve it in 1 ml chloroform-methanol solvent mixture (see below) to prepare a 10 mM solution. Keep the solution, tightly closed, on ice and use within 12 h. Note: Use of this reagent is optional and in most cases this is not necessary. See "Interferences" on page 8 for details.
6. Glass 96 Well Plate: The glass plate is supplied with the 96 well size, and is reusable. The glass plate must be cleaned before use each time. Clean the plate with warm soapy water, and then rinse it with HPLC-grade water, followed by rinsing with acetone. *NOTE: Do not clean the plate with abrasive agents. The plate must be completely dry before use in the assay.*

Solvents

Deoxygenate about 100 ml each of chloroform and methanol (these solvents are not provided as part of the kit) by bubbling nitrogen through the solvents for at least 30 minutes.

Cool part of the deoxygenated chloroform to 0°C and store it on ice for extraction of the samples. Two volumes of chloroform are required for the extraction of one volume of sample.

Mix two volumes of chloroform with one volume of methanol. The solvent mixture is ready for use in the assay. Approximately 1 ml of chloroform-methanol mixture is needed for each assay tube.

PIPETTING HINTS

- When pipetting chloroform or chloroform-methanol only polypropylene or teflon based pipet tips should be used.
- Before pipetting each reagent, equilibrate the pipet tip in that reagent (i.e., fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipet tip to the reagent(s) already in the test tube.

Sample Preparation

Most tissues contain peroxidases (e.g. glutathione peroxidase) that effectively reduce endogenous lipid hydroperoxides to their corresponding alcohols. Peroxidase activity decreases hydroperoxide concentrations to extremely low or undetectable levels in normal tissues. Even under oxidative stress conditions, hydroperoxide production by free radicals must overwhelm the peroxidase defenses before a detectable increase in lipid hydroperoxide concentration will occur. Measurement of lipid hydroperoxides provides a snapshot of the lipid peroxidation level at the time of the assay. Integrated values of lipid peroxidation (lipid peroxidation over time) can be determined more reliably by measuring the 8-isoprostane levels (8-Isoprostane EIA Kit).^{11,12}

Any sample containing lipid hydroperoxides is suitable for this assay. Tissues, cultured cells, plant materials, foods, and biological fluids such as plasma can be used in the assay. Tissues, plant materials, and foods should be homogenized in HPLC-grade water or in buffer containing no transition metal ions before use. Cultured cells should be sonicated in HPLC-grade water or in media containing no transition metal ions before use. Samples should be assayed immediately upon collection. If samples cannot be assayed fresh, then the lipid hydroperoxides should be extracted and the extracts should be stored at -80°C. The extracted lipid hydroperoxides are stable for at least one month at -80°C.

Lipid hydroperoxides must be extracted from the sample into chloroform before performing the assay. The standard Bleigh and Dyer¹³ extraction protocol is not reproducible and hence not suitable for quantitative analysis. In this kit, a deproteination procedure is combined with the extraction of lipid hydroperoxides to achieve quantitative extraction of lipid hydroperoxides. This extraction step ensures the elimination of nearly all interfering substances from the sample. The following is a typical extraction procedure using plasma as the sample:

1. Aliquot a known volume of sample (e.g. 500 µl of plasma) into a glass test tube (12 x 75 mm).
2. Add an equal volume of Extract R saturated methanol (500 µl for this example) to each tube and vortex.
3. Add 1 ml of cold chloroform to each tube and mix thoroughly by vortexing.
4. Centrifuge the mixture at 1500 x g for 5 minutes at 0°C.
5. Collect the bottom chloroform layer by carefully inserting a pasteur pipet along the side of the test tube. Transfer the chloroform layer to another test tube and store on ice. **CAUTION:** *Avoid collecting the middle protein layer or the upper water layer along with the chloroform layer. Any water carried over to the assay tube will interfere with color development. It is not necessary to collect all of the chloroform layer; 700 µl will be sufficient.*

PERFORMING THE ASSAY

- *The final volume of the assay is 1 ml in all tubes.*
- *It is not necessary to use all the reagents at one time. However, a standard curve must be run simultaneously with each set of samples.*
- *If the concentration of lipid hydroperoxides in the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.*
- *It is recommended that the samples and standards be assayed in triplicate.*
- *Prepare the Chromogen (see below) just before its addition to the tubes.*
- *The background absorbance (abs. of Std. A) should be ≤ 0.3 .*

1. Preparation of the standards: Take 24 clean test tubes (glass or polypropylene) and mark them A-H in triplicate. Aliquot the Lipid Hydroperoxide Standard (HP) (vial #3) and chloroform-methanol mixture to each tube as described in Table 1.

Tube	HP Standard (μl)	CHCl ₃ -CH ₃ OH (μl)	Final HP* (nmol)
A	0	950	0
B	10	940	0.5
C	20	930	1.0
D	30	920	1.5
E	40	910	2.0
F	60	890	3.0
G	80	870	4.0
H	100	850	5.0

*This is the final amount of hydroperoxide in the assay tube

Table 1.

2. Add 500 μl † of the chloroform extract of each sample to appropriately labeled glass test tubes. *Note:* Avoid any transfer of water from the extract.
3. Aliquot 450 μl of chloroform-methanol solvent mixture to each test tube.
4. Prepare the Chromogen by mixing equal volumes of FTS Reagent 1 and FTS Reagent 2 in test tube and vortex. The volume of Chromogen to be prepared is dependent on the number of tubes assayed. Calculate 50 μl for each tube (e.g. 24 x 50 μl for the tubes of the standard curve).
5. Add 50 μl of the freshly prepared Chromogen to each assay tube and mix well on a vortex mixer. Close the test tubes tightly with polypropylene caps.
6. Keep the assay tubes at room temperature for 5 min.

There are two methods of completing this assay, either using a spectrophotometer to test each sample separately (see step 7) or when using the glass 96-well plate, the values can be read with a plate reader (see steps 8-10).

7. Measure the absorbance of each tube at 500 nm using either glass or quartz 1 ml cuvettes. Use chloroform-methanol solvent mixture for blank if the spectrophotometer requires it.

OR

8. Transfer 300 μl from each tube into the 96-well plate following the configuration shown in Figure 1 below. (There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in triplicate is given.) To avoid evaporation from the wells, you may cover the plate with aluminum foil as you aliquot the samples. *NOTE: Do not use plastic plate covers, the solvent will dissolve the cover. Do not shake the plate on the table top, it will scratch the bottom of the plate.*

9. Read the absorbance at 500 nm using a 96 well plate reader.

10. The 96-well plate can be reused, see Pre-Assay Preparation (page 3) for instructions on how to clean the plate.

Note: The color is stable for two hours. If the test tubes are not closed tightly, evaporation of solvent will result in change of absorbance.

†Volume of the extract used for the assay can be changed depending on the concentration of hydroperoxide. However, adjust the volume of the chloroform-methanol solvent mixture accordingly to a final volume of 950 µl before the addition of the chromogen.

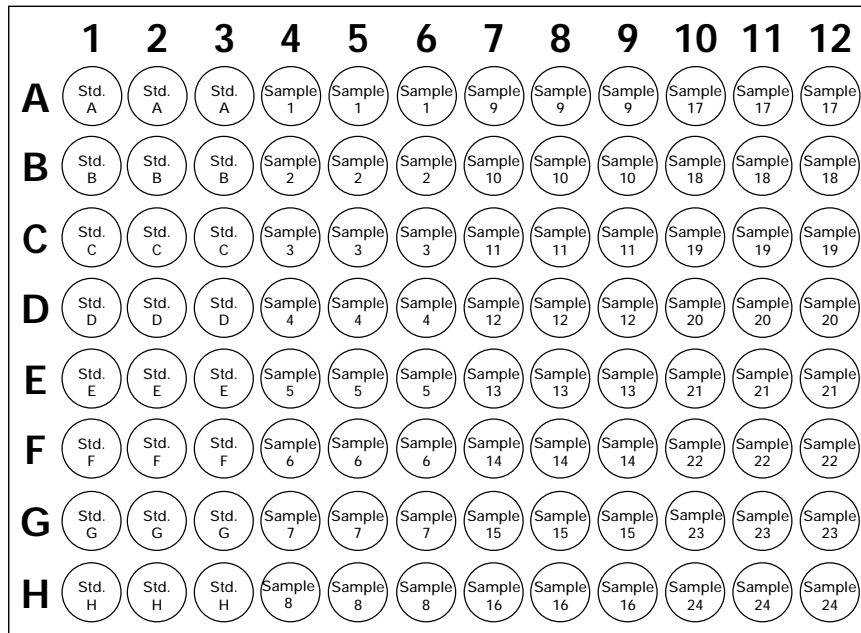


Figure 1. Plate configuration

CALCULATING THE RESULTS

1. Calculate the average absorbances of each standard and sample.
2. Subtract the average absorbance of standard A from itself and all other standards and samples.
3. Plot the corrected absorbance of standards (from step 2 above) as function of final hydroperoxide value from Table 1. See Figure 2 for a typical standard curve using a spectrophotometric assay. See Figure 3 for a typical standard curve using a 96 well plate format.

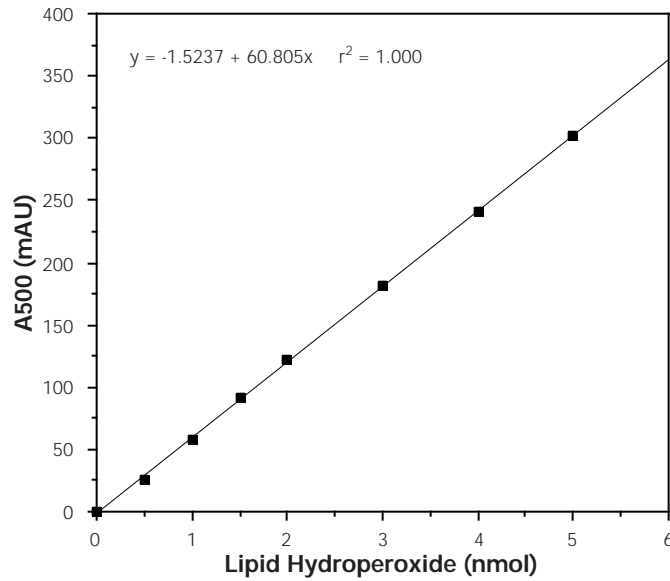


Figure 2. Typical standard curve for the spectrophotometric assay.

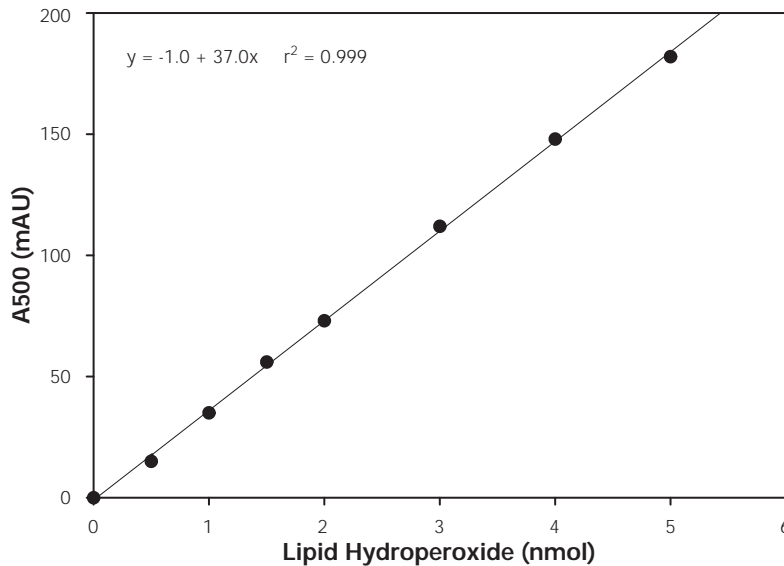


Figure 3. Typical standard curve for the 96 well plate format

4. Calculate the hydroperoxide values of the sample tubes (HPST) using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

$$\text{HPST (nmol)} = (\text{sample absorbance} - \text{y-intercept})/\text{slope}$$

5. Calculate the concentration of hydroperoxide in the original sample as shown below:

$$\text{Volume of extract used for the assay} = \text{VE (ml)}$$

$$\text{Volume of the original sample used for extraction} = \text{SV (ml)}$$

$$\text{Conc. hydroperoxide in sample } (\mu\text{M}) = \frac{\text{HPST}}{\text{VE}} \times \frac{1 \text{ ml}}{\text{SV}}$$

INTERFERENCES

The extraction protocol used in the sample preparation eliminates nearly all possible interfering substances. However, if the extracts exhibit strong absorbance at 500 nm (before the addition of chromogen), they may not be suitable for this assay. The color of commonly used samples such as plasma do not interfere with the assay. Any non-hydroperoxide generated color in the assay can be measured by first reducing the hydroperoxides with triphenylphosphine. Triphenylphosphine reduces hydroperoxides very efficiently and eliminates the color development due to hydroperoxides in the assay. The resultant absorbance after treating the sample with triphenylphosphine represents the background absorbance which can be subtracted from the sample data to correct for any 500 nm absorbance not due to hydroperoxides. The procedure is as follows:

1. Perform extraction of samples as described under "Sample Preparation" (see page 4) in duplicate.
2. Add 10 μl of chloroform-methanol solvent mixture to one extract and keep it on ice.
3. Add 10 μl of triphenylphosphine solution to the second extract, mix well and let it stand at room temperature for at least 5 min. If the sample is not used immediately, store it on ice until use.
4. Aliquot the required amount of extract from each tube as described in step 2 under "Performing the Assay" (see page 5) and proceed with the rest of the assay.
5. Multiply the absorbance values of the triphenylphosphine containing samples by 1.28 (to correct for the effect of triphenylphosphine on the chromogen) and use these values as blanks for the corresponding samples. The remaining calculations should be identical to those described above.

TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates.

Cause: Poor pipetting/technique.

Problem: No color development.

Cause: Both reagents are not added to prepare the chromogen or hydroperoxide standard decomposed.

Solution: Make sure to add both reagents to prepare the chromogen. Obtain a fresh hydroperoxide standard.

Problem: No color in the sample above the background.

Cause: Concentration of lipid hydroperoxides in the sample is too low ($<0.25 \mu\text{M}$).

Solution: Use more volume of the extract for the assay while keeping the final assay volume at 1 ml. Use more sample for extraction.

Problem: Absorbance value of the sample higher than the highest point of the standard curve.

Cause: Sample is too concentrated.

Solution: Use less volume of the extract while keeping the final assay volume to 1 ml. Alternatively, dilute the extract with chloroform-methanol solvent mixture.

Problem: Background absorbance is higher than 0.3.

Cause: The solvents were not degassed long enough.

Solution: Make sure to degas the chloroform and methanol with nitrogen for at least 30 minutes.

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NOTES