Lipid Hydroperoxide (LPO) Assay Kit

Introduction

Lipid peroxidation is one of the important indicators to evaluate the level of oxidative stress, which results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Traditionally, lipid peroxidation is mainly detected by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the degradation products of polyunsaturated fatty acids (PUFAs). There are limitations to using both methods for detection, as only lipid peroxidation from PUFAs produces these products, which can lead to inaccurate results. Given the limitations of these assays, direct measurement of lipid peroxides is a better option.

Lipid Hydroperoxide (LPO) Assay Kit is an optimized kit for the direct detection of lipid hydroperoxides using a redox reaction with ferrous ions. Lipid hydroperoxides are highly unstable and readily react with ferrous ions to form ferric ions, which can be detected by thiocyanate ions as chromogenic agents. However, many samples may contain ferric ions, which can interfere with detection. The kit is optimized to extract lipid hydroperoxides from samples with chloroform, eliminating any interference caused by hydrogen peroxide or endogenous ferric ions in the samples.

Components and Storage

Components	K2265-100 T
LPO Assay FTS Reagent 1	3 mL
LPO Assay FTS Reagent 2	3 mL
Lipid Hydroperoxide Standard	3.5 mL
LPO Assay Extract R	1 vial
LPO Assay Triphenylphosphine	1 vial
Store Lipid Hydroperoxide Standard at -80°C, and the remaining reagents should be stored at 4°C. This kit is stable for 1 year.	

Protocol

1. **Reagents not provided in this kit**

   1) Chloroform

   2) Methanol

   3) 96-well glass plates or chloroform-resistant plates

2. Reagents preparation

1) Keep Lipid Hydroperoxide Standard on ice during the experiment.

2) Extract R saturated solution preparation: Weigh about 100 mg LPO Assay Extract R, add 15 mL of methanol and vortex thoroughly for about 2 min. The solution will be cloudy, and most of the solid remain undissolved. Use the Extract R saturated solution within 2 h.

3) Chloroform/methanol deoxygenation: Prepare 100 mL of chloroform and 100 mL of methanol, and bubble the solvent for at least 30 minutes with nitrogen respectively. For best results, fresh chloroform is recommended. Partially deoxygenated chloroform is cooled to 0°C to extract the sample.

4) Preparation of chloroform-methanol solution: Chloroform and methanol are mixed evenly according to the volume ratio of 2:1 to make the chloroform-methanol solution.

5) LPO Assay Triphenylphosphine solution preparation (optional): Dissolve 2.6 mg LPO Assay Triphenylphosphine in 1 mL of chloroform-methanol solution to make a 10 mM stock solution. Keep the stock solution on ice and use it within 12 h.

***Note:** LPO Assay Triphenylphosphine is not required in most cases. See step 9 for details.

3. Samples preparation:

1) Cultured cells: Sonicate in HPLC-grade water or a medium containing no transition metal ions before use.

2) Tissues/Plant/Food: Homogenize in HPLC-grade water or a medium containing no transition metal ions before use.

4. Lipid hydroperoxides extraction (plasma as an example):

1) Aliquot a known volume of sample (e.g., 500 µL) of plasma into a glass tube.

2) Add an equal volume of Extract R saturated solutions to tube and vortex.

3) Add 1 mL of pre-chilled chloroform to each tube and vortex to mix well.

4) Centrifuge at 0°C, 1500 g for 5 min.

5) Carefully insert a pasteur pipette or syringe needle along the side of the tube to collect the chloroform layer at the bottom. Transfer the chloroform layer to another tube and keep it on ice. It is not necessary to collect all the chloroform layers, in this case 700 µL is sufficient.

***Note:** Avoid collecting the middle protein layer or the upper water layer when collecting the bottom chloroform layer. Water carried into a new tube can interfere with subsequent color reactions. If it is not immediately detectable, the extracted lipid hydroperoxide can be stored at -80°C, which is generally stable for 1 month.

5. Standard preparation:

Prepare 24 tubes (glass or polypropylene) and label them as groups A-H in triplicate. Refer to the following table to prepare:

	Standard (μL)	chloroform-methanol solution (μL)	Final hydroperoxide (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

6. **LPO assay**

1) Add 500 μL of the chloroform extract of samples to glass tubes. Then add 450 μL of chloroform-methanol solution to tubes and mix.

***Note:** The amount of chloroform extracted sample can be adjusted according to the experiment, as long as the final volume of the chloroform-methanol mixture is 950 μL.

2) Prepare the chromogen: Mix equal volumes of LPO Assay FTS Reagent 1 and LPO Assay FTS Reagent 2 and vortex to make the chromogen. It can be prepared according to the amount required for the experiment. 50 μL of chromogen is needed for each sample. Chromogenic agents are recommended to be freshly prepared.

3) Add 50 μL of freshly prepared chromogen to each sample and standard tube, mix well, and close the tubes tightly with polypropylene caps.

4) Incubate for 5 minutes at room temperature.

7. **Detection**: The following two methods can be chosen.

1) Measure the absorbance value of each sample at 500 nm by spectrophotometer. A chloroform-methanol solution can be used as a blank control if needed. Try to complete the test within 2 hours.

2) Transfer 300 μL from each tube to a 96-well glass plate (or chloroform-resistant) and measure with a microplate reader at 500 nm. Try to complete the test within 2 hours. To avoid evaporation of the solution, the plate can be covered with aluminum foil.

***Note:** Do not use plastic plates or covers.

8. Analysis:

The standard curve：y=ax+b

The amount of LPO in the sample（nmol）=HPST=(A_{500} -b)÷a

The amount of LPO in the original sample（μM）= (HPST÷V_1) ×(V_2÷V_3)

***Note:** y: the detection value of each standard well; x: the concentration of the wells of each standard; A_{500}: Measured value of the sample at 500 nm; V_1: The volume of the sample used in the test, mL; V_2: volume of reaction system at the time of detection, 1mL; V_3: Original sample volume used for extraction, mL

9. Triphenylphosphine treatment（optional）:

Most of the extracted samples do not have strong interfering substances. However, if the extracted sample has strong absorption at 500 nm before adding chromogen, it indicates that there are strong interfering substances in the sample. To rule out the influence of interference on experimental results, Triphenylphosphine treatment can be used. Triphenylphosphine is very effective at reducing lipid hydroperoxides, thereby removing color development caused by lipid hydroperoxides from the sample. The measured value after Triphenylphosphine treatment is the background value and can be subtracted to correct for the true detection value in the sample.

1) In step 4, divide the samples into two aliquots and extract them separately.
2) Add 10 μL of the chloroform-methanol solution to one aliquot and keep it on ice.
3) Add 10 μL of LPO Assay Triphenylphosphine solution to another aliquot, mix well and let it stand for 1 h at room temperature. If the test is not immediate, it can be kept on ice until use.
4) Aliquot a certain amount of extract from each tube as described in step 6 and proceed with the rest of the assay.
5) Multiply the absorbance of the triphenylphosphine-treated samples by 1.28 (to correct for the effect of Triphenylphosphine on the chromogen). And use these values as blanks for the corresponding samples.

Note

1. The background absorbance of this kit is generally less than 0.3.
2. When pipetting chloroform or chloroform-methanol, use polypropylene or Teflon-based tips. Some tips manufacturers may add slip or antistatic agents to the tips, if the background absorbance is greater than 0.3, it may be that there is interference in the tips, which needs to be excluded.
3. The dynamic range of this kit is 0.5-5 nmol hydroperoxide per tube. If the sample concentration exceeds this range, it is recommended that the sample be diluted before testing.
4. For research use only. Not to be used in clinical diagnostic or clinical trials.

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