Lipoxygenase Inhibitor Screening Assay Kit

Catalog Number KA1329

96 assays

Version: 05

Intended for research use only

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Introduction

Background

Lipoxygenases (LOs) are non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen to fatty acids containing a *cis,cis*-1,4-pentadiene system. The initial product of this reaction is a 4-hydroperoxy *cis-trans*-1,3-conjugated pentadienyl moiety within the unsaturated fatty acid.[1,2] The three main LO enzymes are designated 5-, 12-, and 15-LO based on the position of the introduced hydroperoxide. Linoleate and arachidonate are the common substrates for LOs in plants and animals.

Principle of the Assay

Lipoxygenase Inhibitor Screening Assay Kit detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified LO. The detection reaction is equally sensitive to hydroperoxides at various positions within the fatty acid, and will work with fatty acids of any carbon length. It is thus a general detection method for LO, and can be used to screen libraries of compounds for those which inhibit LO enzymes.

General Information

Materials Supplied

List of component

Item	Quantity/Size
Lipoxygenase Inhibitor Screening Assay Buffer (10X)	1 vial
Developing Reagent 1	1 vial
Developing Reagent 2	1 vial
15-Lipoxygenase Standard	1 vial
Arachidonic Acid (Substrate)	1 vial
Linoleic Acid (Substrate)	1 vial
Potassium Hydroxide	1 vial
96-Well Solid Plate (Colorimetric Assay)	1 plate
96-Well Cover Sheet	1 cover

Storage Instruction

Item	Storage
Lipoxygenase Inhibitor Screening Assay Buffer (10X)	4°C
Developing Reagent 1	4°C
Developing Reagent 2	4°C
15-Lipoxygenase Standard	4°C
Arachidonic Acid (Substrate)	-20°C
Linoleic Acid (Substrate)	-20°C
Potassium Hydroxide	4°C
96-Well Solid Plate (Colorimetric Assay)	Room temperature
96-Well Cover Sheet	Room temperature

Materials Required but Not Supplied

- ✓ A plate reader capable of measuring absorbance between 490-500 nm
- ✓ Adjustable pipettors and a repeat pipettor
- ✓ A source of pure water. Glass distilled water or HPLC-grade water is acceptable
- ✓ Hydrogen peroxide (420 µM)

Precautions for Use

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- Pipetting Hints

✓ It is recommended that a repeating pipettor be used to deliver substrate and Chromogen to the wells.
✓ Use different tips to pipette sample, substrate, and Chromogen.
✓ Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
✓ Do not expose the pipette tip to the reagent(s) already in the well.

- General Information

✓ The final volume of the assay is 210 µL in all the wells.
✓ It is not necessary to use all the wells on the plate at one time.
✓ If the appropriate inhibitor dilution is not known, it may be necessary to assay at several dilutions.
✓ Use the diluted Assay Buffer in the assay.
✓ It is recommended that samples be assayed at least in duplicate (triplicate preferred).
✓ The background absorbance (absorbance of the blank wells) should be <0.22.

Assay Protocol

Reagent Preparation

- Lipoxygenase Inhibitor Screening Assay Buffer (10X) - Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Assay Buffer (0.1 M Tris-HCl, pH 7.4) should be used for dilution of samples and the 15-LO standard prior to assaying. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.
- Developing Reagent 1 - The reagent is ready to use as supplied.
- Developing Reagent 2 - The reagent is ready to use as supplied.
- Chromogen - Prepare the Chromogen prior to use by mixing equal volumes of Developing Reagent 1 and Developing Reagent 2 in a test tube and vortexing. The volume of Chromogen to be prepared is dependent on the number of wells assayed. Calculate 100 µL for each well. Use the Chromogen within one hour.
- 15-Lipoxygenase Standard - A solution of 15-LO (soybean) is supplied as a positive control. Transfer 10 µL of the supplied enzyme to another vial and dilute with 990 µL of diluted Assay Buffer prior to use, store on ice, and use within one hour. A 90 µL aliquot of the enzyme per well causes a final absorbance of approximately 0.19 under the standard assay conditions.
- Arachidonic Acid (Substrate) - This vial contains a solution of arachidonic acid in ethanol and should be stored at -20°C when not being used. Transfer 25 µL of the supplied substrate to another vial, add 25 µL of Potassium Hydroxide, vortex, and dilute with 950 µL of HPLC-grade water to achieve a final concentration of 1 mM. Use the prepared arachidonic acid solution within 30 minutes. A 10 µL aliquot will yield a final concentration of 100 µM in the wells. *NOTE: You can use either arachidonic or linoleic acid in the assay. You do not have to use both.*
- Linoleic Acid (Substrate) - This vial contains a solution of linoleic acid in ethanol and should be stored at -20°C when not being used. Transfer 25 µL of the supplied substrate to another vial, add 25 µL of Potassium Hydroxide, vortex, and dilute with 950 µL of HPLC-grade water to achieve a final concentration of 1 mM. Use the prepared linoleic acid solution within 30 minutes. A 10 µL aliquot will yield a final concentration of 100 µM in the wells. *NOTE: You can use either arachidonic or linoleic acid in the assay. You do not have to use both.*
- Potassium Hydroxide - This vial contains 0.1 M potassium hydroxide (KOH). The reagent is ready to use as supplied.
- Plate setup - There is no specific pattern for using the wells on the plate. However, it is necessary to have some wells (at least two) designated as non-enzymatic controls (blanks). The absorbance of these wells must be subtracted from the absorbance measured in the sample wells. We suggest that you have at least two wells designated as positive controls. A typical layout of samples to be measured in duplicate is shown in Figure 1.

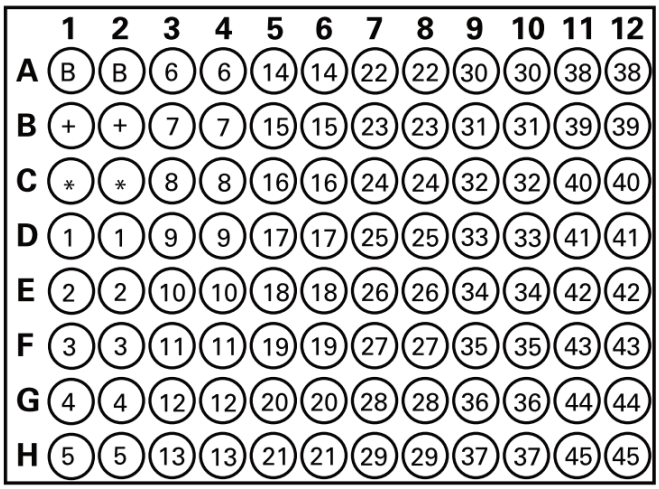

Figure 1. Sample plate format

Sample preparation

- Cell lysates and tissue homogenates contain peroxidases (e.g., glutathione peroxidase) that will reduce the lipid hydroperoxides generated in the assay, resulting in a very low signal. To achieve the most accurate results, we recommend screening purified LOs (5-, 12-, or 15-LO) with this assay. The sample must be free of particulates to avoid interferences in the absorbance measurement. Phosphates, EDTA, transition metal ions, thiols, and any endogenous LO inhibitors must be removed from the samples before performing the assay (extensive dialysis or concentrating and reconstituting in a Tris Buffer several times will eliminate most of the interfering substances of small molecular size).
- If the enzymes are too dilute, they can be concentrated using a membrane filter with a molecular weight cut-off of 30,000 Da (such as an Amicon centrifuge concentrator).
- Cyclooxygenases will not be measured by this assay. If you are concerned that the activity seen in your sample is due to a cyclooxygenase (COX-1 or COX-2), then add a non-selective COX inhibitor (i.e., Indomethacin) as a control.

Assay Procedure

1. Blank Wells - add 100 µL of Assay Buffer to at least two wells.
2. Positive Control Wells (15-LO Standard) - add 90 µL 15-LO and 10 µL of Assay Buffer to at least two wells.
3. 100% Initial Activity Wells - add 90 µL of lipoxygenase enzyme and 10 µL of solvent (the same solvent used to dissolve the inhibitor) to two wells. The 100% initial activity wells should result in approximately 10 nmol/min/mL of activity.
4. Inhibitor Wells - add 90 µL of lipoxygenase enzyme and 10 µL of inhibitor* to two wells.
5. Initiate the reaction by adding 10 µL of substrate (either Arachidonic or Linoleic Acid) to all the wells. Place the 96-well plate on a shaker for at least five minutes.

6. Add 100 µL of Chromogen to each well to stop enzyme catalysis and develop the reaction. Cover with a plate cover and place the 96-well plate on a shaker for five minutes.
7. Remove the cover and read the absorbance at 490-500 nm using a plate reader.

**Inhibitors can be dissolved in diluted Assay Buffer, methanol, DMSO, or ethanol. The inhibitor should be added to the assay in a final volume of 10 µL before initiating with substrate. In the event that the appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made.*

- Interferences

✓ Culture Medium and Buffers

All buffers and medium should be tested for high background absorbances before doing any experiments. If the initial background absorbances are higher than 0.22 absorbance units then the samples should be diluted in diluted Assay Buffer or HPLC-grade water before performing the assay. Phosphate, HEPES, and EDTA interfere with the Chromogen and will result in no enzyme activity. Tris, borate, and EGTA work fine in the assay. DMEM (Dulbecco's Modified Eagles Medium) and MEM (Minimum Essential Medium Eagle) exhibit high background absorbances and should not be used in the assay. However, F-12 (Ham Nutrient Mixture) does not interfere with the assay.

✓ Thiols and Transition Metal Ions

Buffers containing thiols (i.e., glutathione, cysteine, dithiothreitol, or 2-mercaptoethanol) and transition metal ions (i.e., Fe, Mn, or Cu) will exhibit high background absorbances and interfere with LO activity determination. Extensive dialysis will eliminate most of the interfering substances of small molecular size.

✓ Solvents

Inhibitors can be dissolved in methanol, ethanol, or DMSO. The inhibitor should be added to the assay in 10 µL.

✓ Inhibitors

LO inhibitors should be tested for assay interference by following the protocol outlined below:

1. Blank Wells - add 100 µL of diluted Assay Buffer to at least two wells.
2. Blank Wells plus Inhibitor Wells - add 90 µL of diluted Assay Buffer and 10 µL of inhibitor to at least two wells.
3. Hydrogen Peroxide (H_2O_2) Wells - add 90 µL of diluted Assay Buffer and 10 µL of 420 µM H_2O_2 (not supplied in the kit) to at least two wells.
4. Hydrogen Peroxide (H_2O_2) plus Inhibitor Wells - add 80 µL of diluted Assay Buffer, 10 µL of 420 µM H_2O_2, and 10 µL of inhibitor to at least two wells.
5. Initiate the reaction by adding 10 µL of substrate (either Arachidonic or Linoleic Acid) to all the wells. Place the 96-well plate on a shaker for five minutes.

6. Add 100 µL of Chromogen to each well and develop the reaction. Cover with a plate cover and place the 96-well plate on a shaker for five minutes.
7. Remove the cover and read the absorbance at 500 nm using a plate reader.
   Note: The blank plus inhibitor wells should not exhibit an absorbance >0.22. If the absorbance is above 0.22, then try diluting with diluted Assay Buffer or solvent the inhibitor is dissolved in. The H_2O_2 wells and the H_2O_2 plus inhibitor wells should exhibit approximately the same absorbance. If the H_2O_2 plus inhibitor wells exhibit an absorbance higher or lower than the H_2O_2 wells, then the inhibitor is interfering with the assay. Try diluting the inhibitor with more diluted Assay Buffer or solvent.

Data Analysis

Calculation of Results

1. Determine the average absorbance of the blank, 100% initial activity (IA), and inhibitor wells.
2. Subtract the average absorbance of the Blank from the average absorbance of the 100% IA and inhibitor wells.
3. Determine the percent inhibition or percent IA for each inhibitor using one of the following equations.

$$\%\,\text{Inhibition} = \left[\frac{\text{IA} - \text{Inhibitor}}{\text{IA}}\right] \text{x}\, 100$$

$$\%\,\text{IA} = \frac{\text{Inhibitor}}{\text{IA}} \text{x}\, 100$$

4. Graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). Examples of 5- and 15-LO inhibition by nordihydroguaiaretic acid (NDGA).

OPTIONAL:

If you want to determine the LO activity, use the following formula. The reaction rate at 500 nm can be determined using the chromagen extinction coefficient of 9.47 mM^{-1}. The extinction coefficient has been adjusted for the pathlength of the solution in the well. One unit of enzyme utilizes one µmol of arachidonic or linoleic acid per minute at 25°C.

$$\text{Lipoxygenase Activity (µmol/min/mL)} = \frac{A_{500}(\text{sample}) \quad A_{500}(\text{Blank})}{9.47\text{mM}^{-1} \text{ x } 5\text{min.}} \text{ x } \frac{0.21\text{mL}}{0.09\,\text{mL}} \text{ x sampledilution}$$

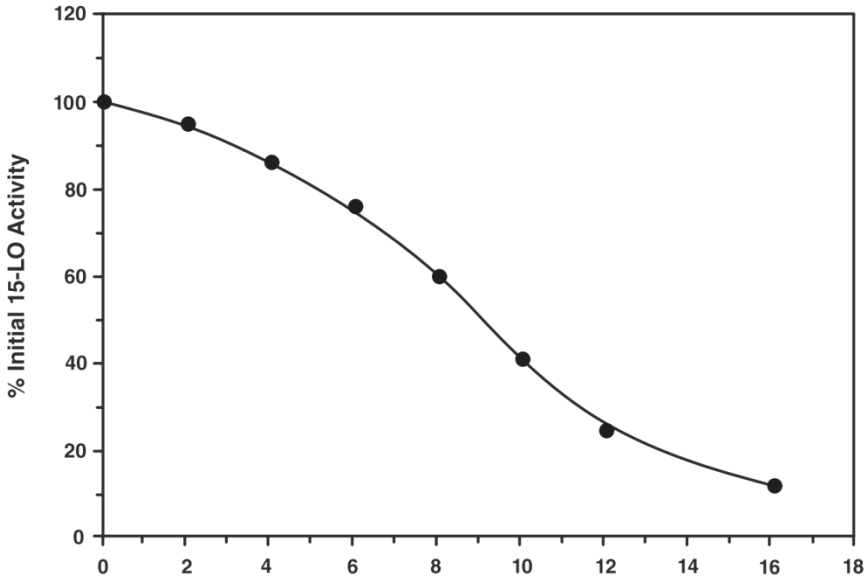

Figure 2. Inhibition of soybean 15-lipoxygenase by NDGA (IC_{50} = 9 µM).

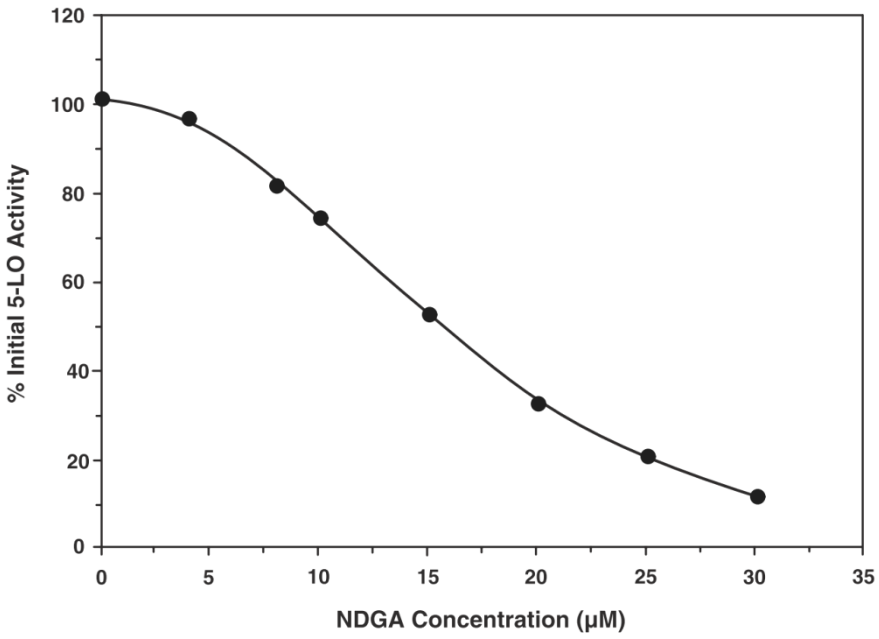

Figure 3. Inhibition of potato 5-lipoxygenase by NDGA (IC_{50} = 15 µM).

Performance Characteristics

- Sensitivity

  Under the standardized conditions described in this booklet, samples containing LO activity between 1-10 nmol/min/mL can be assayed without further dilution or concentration. The assay will detect 0.5-5 nmol of lipid hydroperoxides.

- Linearity of the Assay

  The following graphs exhibit the linearity of the assay using soybean 15-LO.

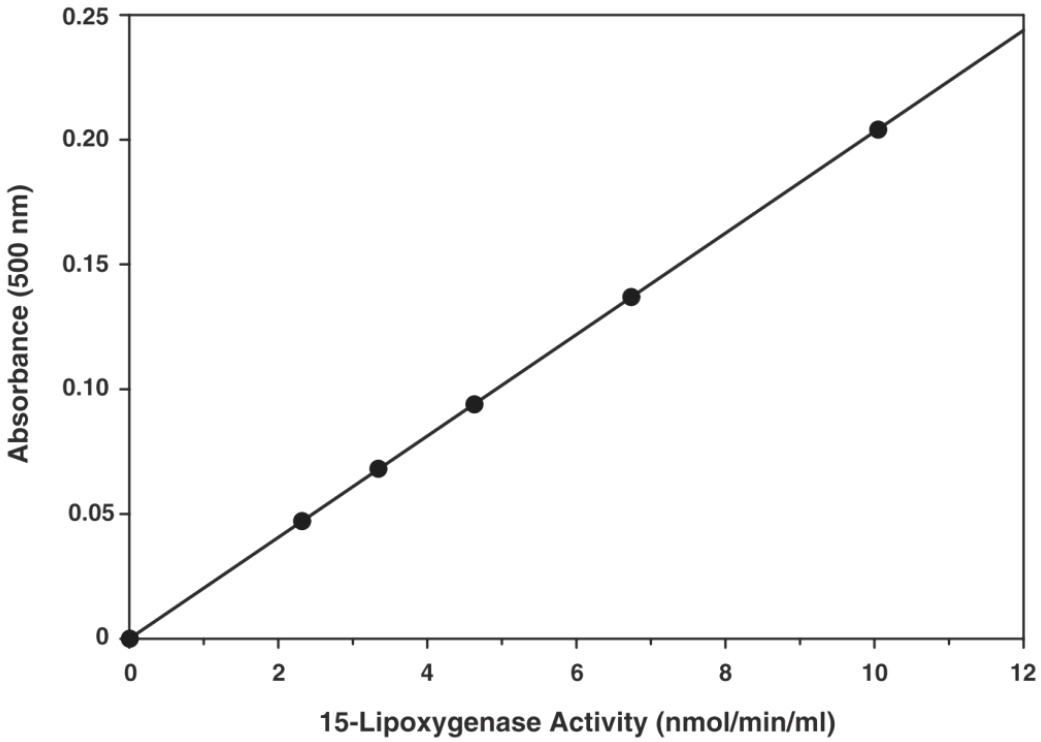

Figure 4. Linearity of the assay using linoleic acid as the substrate

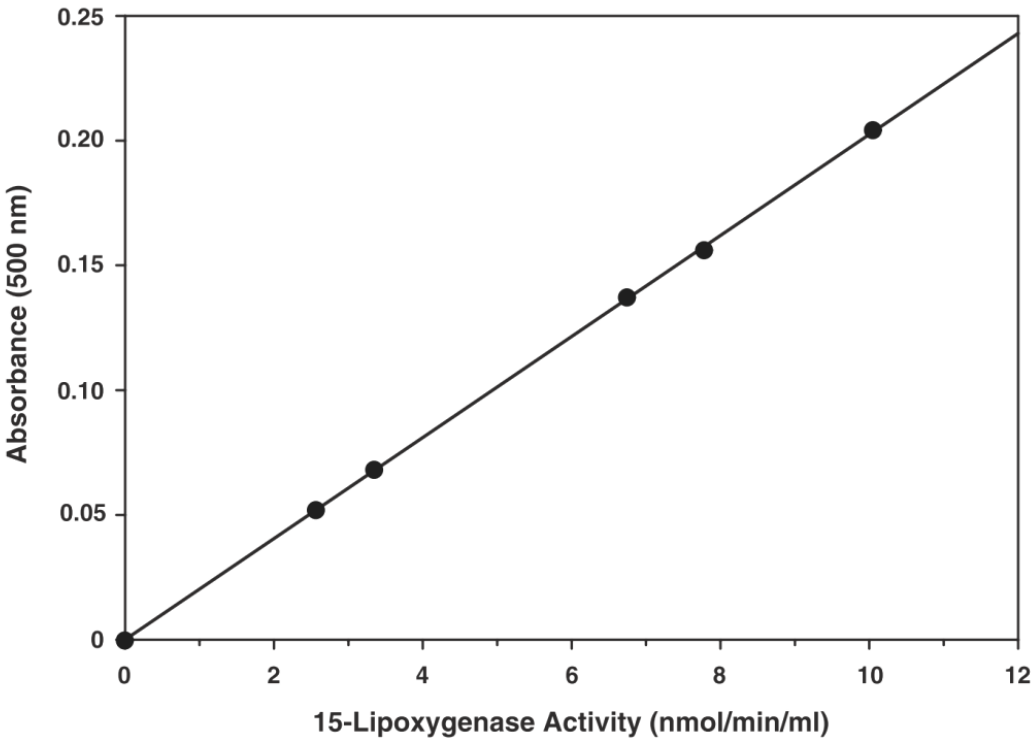

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Figure 5. Linearity of the assay using arachidonic acid as the substrate.

Note: The same results are obtained with 5-LO (potato) and 12-LO (porcine leukocyte).

Resources

Trouble shooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No color development	A. Enzyme, substrate, or Chromogen was not added to the well(s); enzyme activity was too low B. Something is interfering with the Chromogen.	Make sure to add all components to the wells and standardize the assay with the 15-LO standard; concentrate the enzyme so that the activity falls within the range of the assay; see the Interference section to confirm that the enzyme does not contain something that will effect the performance of the assay
High background abosorbance (>0.22)	There is something interfering with the assay	See the Interference section

References

1. Gaffney, B.J. Lipoxygenases: Structural principles and spectroscopy. Annu. Rev. Biophys. Biomol. Struct. 25, 431-459 (1996).
2. Yamamoto, S. Mammalian lipoxygenases: Molecular structures and functions. Biochim. Biophys. Acta 1128, 117-131 (1992).

Plate Layout

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