Lipoxygenase Inhibitor Screening Assay Kit

Item No. 760700

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
760710	Lipoxygenase Inhibitor Screening Assay Buffer (10X)	1 vial	4°C
760711	Developing Reagent 1	1 vial	4°C
760712	Developing Reagent 2	1 vial	4°C
760714	15-Lipoxygenase Standard	1 vial	4°C
760715	Arachidonic Acid (substrate)	1 vial	-20°C
760716	Linoleic Acid (substrate)	1 vial	-20°C
760713	Potassium Hydroxide	1 vial	4°C
760717	Nordihydroguaiaretic Acid (NDGA) Positive Control Inhibitor	1 vial	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, see page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 490-500 nm
2. Adjustable pipettors and a repeating pipettor
3. A source of pure water. Glass distilled water or HPLC-grade water is acceptable
4. Hydrogen peroxide (420 µM)
5. Methanol to resuspend the inhibitor

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.

About This Assay

Cayman's 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.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Lipoxygenase Inhibitor Screening Assay Buffer (10X) - (Item No. 760710)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final 1X 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 1X Assay Buffer is stable for at least two months.

2. **Developing Reagent 1 - (Item No. 760711)**

   The reagent is ready to use as supplied.

3. **Developing Reagent 2 - (Item No. 760712)**

   The reagent is ready to use as supplied.

4. **Chromogen**

   Prepare the Chromogen prior to use by mixing equal volumes of Developing Reagent 1 (Item No. 760711) and Developing Reagent 2 (Item No. 760712) 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.

5. **15-Lipoxygenase Standard - (Item No. 760714)**

   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 1X Assay Buffer prior to use, store on ice, and use within one hour.

6. **Arachidonic Acid (substrate) - (Item No. 760715)**

   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 (Item No. 760713), vortex, and dilute with 950 µl of HPLC-grade water to achieve a working concentration of 1 mM. Use the prepared arachidonic acid solution within 30 minutes. A 10 µl aliquot will yield a reaction concentration of 91 µM in the wells. *NOTE: You can use either arachidonic or linoleic acid in the assay. You do not have to use both.*

7. **Linoleic Acid (substrate) - (Item No. 760716)**

   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 (Item No. 760713), vortex, and dilute with 950 µl of HPLC-grade water to achieve a working concentration of 1 mM. Use the prepared linoleic acid solution within 30 minutes. A 10 µl aliquot will yield a reaction concentration of 91 µM in the wells. *NOTE : You can use either arachidonic or linoleic acid in the assay. You do not have to use both.*

8. **Potassium Hydroxide - (Item No. 760713)**

   This vial contains 0.1 M potassium hydroxide (KOH). The reagent is ready to use as supplied.

9. **NDGA Positive Control Inhibitor - (Item No. 760717)**

   This vial contains the non-selective lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) in DMSO. Dilute the NDGA stock solution with 500 µl of 1X Assay Buffer to make a 1.1 mM stock. The addition of 10 µl to the assay yields a final concentration of 100 µM inhibitor in the well. *NOTE: Alternatively, inhibitors can be dissolved in methanol or dimethylsulfoxide (DMSO), however slightly decreased enzyme activity was observed with DMSO. Dimethylformamide (DMF) and ethanol dramatically reduce enzyme activity and thus they are not recommended for dissolving inhibitors.*

Enzyme 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 should 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, Item No. 70270) as a control. *NOTE: Cayman's 5-LO (human recombinant, Item No. 60402) will not work in the assay. Follow the directions on the insert for diluting Cayman's 5-LO (potato) Screening Enzyme (Item No. 60401) prior to assaying. Dilute Cayman's 15-LO (soybean P1; Item No. 60700) 1:2,000 with diluted Assay Buffer (Item No. 760710) prior to assaying.*

ASSAY PROTOCOL

Plate Set Up

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. We also suggest you record the contents of each well on the template sheet provided (see page22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	6	6	14	14	22	22	30	30	38	38
B	+	+	7	7	15	15	23	23	31	31	39	39
C	*	*	8	8	16	16	24	24	32	32	40	40
D	1	1	9	9	17	17	25	25	33	33	41	41
E	2	2	10	10	18	18	26	26	34	34	42	42
F	3	3	11	11	19	19	27	27	35	35	43	43
G	4	4	12	12	20	20	28	28	36	36	44	44
H	5	5	13	13	21	21	29	29	37	37	45	45

B - Blank
+ - Positive Control (15-LO Standard)
* - 100% Initial Activity wells
1-45 - Inhibitor wells

Figure 1. Sample plate format

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 1X 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.

Performing the Assay

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 at least two wells.
4. **Inhibitor Wells -** add 90 µl of lipoxygenase enzyme and 10 µl of inhibitor wells. NDGA Positive Control Inhibitor can be used as a positive control in the assay. *NOTE: Inhibitors can be dissolved in 1X Assay Buffer, methanol, or DMSO. Slightly reduced enzyme activity was seen with DMSO. Ethanol and DMF dramatically reduce enzyme activity and thus they are not recommended for dissolving inhibitors. The inhibitor should be added to the assay in a final volume of 10 µl before initiating with substrate.*

Well	1X Assay Buffer	15-LO	Solvent	Inhibitor
Blank	100 µl	-	-	-
Positive Control	10 µl	90 µl	-	-
100% Initial Activity	-	90 µl	10 µl	-
Inhibitor	-	90 µl	-	10 µl

5. Incubate for five minutes at room temperature.
6. 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 ten minutes.
7. 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.
8. Remove the cover and read the absorbance at 490-500 nm using a plate reader.

ANALYSIS

Calculations

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.

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

$$\% \text{ IA} = \frac{\text{Inhibitor}}{\text{IA}} \times 100$$

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 soybean 15-LO inhibition by nordihydroguaiaretic acid (NDGA) is shown on page 15.

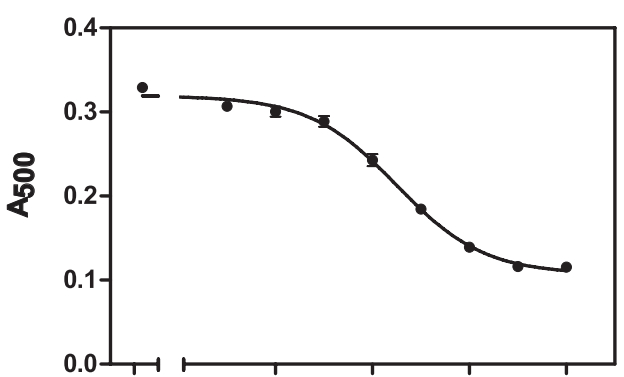


Figure 2. Inhibition of soybean 15-lipoxygenase by NDGA (IC_{50} = 1.6 µM). Arachidonic Acid was used as the substrate in this experiment.

Performance Characteristics

Sensitivity:

Under the standard 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.

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay,[3] which is calculated using the equation below.

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{| \mu_{c+} - \mu_{c-} |}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z´ factor is 1.0. A robust assay has a Z´ factor >0.5. The Z´ factor for Cayman's Lipoxygenase Inhibitor Screening Assay Kit was determined to be 0.88.

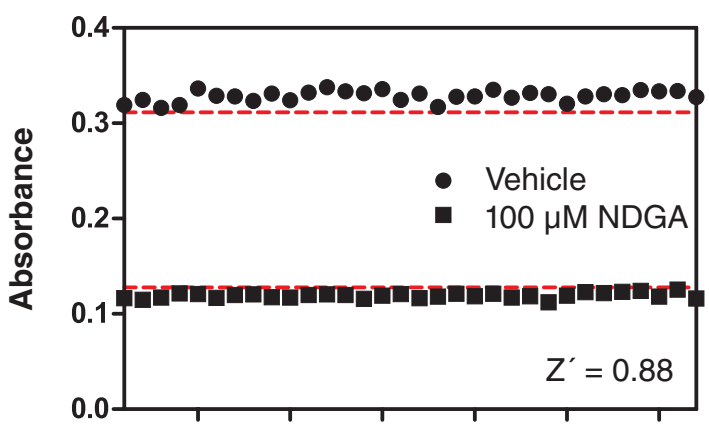


Figure 3. Typical Z´ data for the Lipoxygenase Inhibitor Screening Kit. Data are shown from wells of both positive and negative controls prepared as described in the kit booklet. The calculated Z´ factor from this experiment was 0.88. The red lines correspond to three standard deviations from the mean for each control value.

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 1X 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 or DMSO. However, reduced enzyme activity was observed in the presence of 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 1X Assay Buffer to at least two wells.
2. **Blank Wells plus Inhibitor Wells -** add 90 µl of 1X Assay Buffer and 10 µl of inhibitor to at least two wells.
3. **Hydrogen Peroxide (H_2O_2) Wells -** add 90 µl of 1X 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 1X 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 Acid or Linoleic Acid) to all the wells. Place the 96-well plate on a shaker for ten 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.

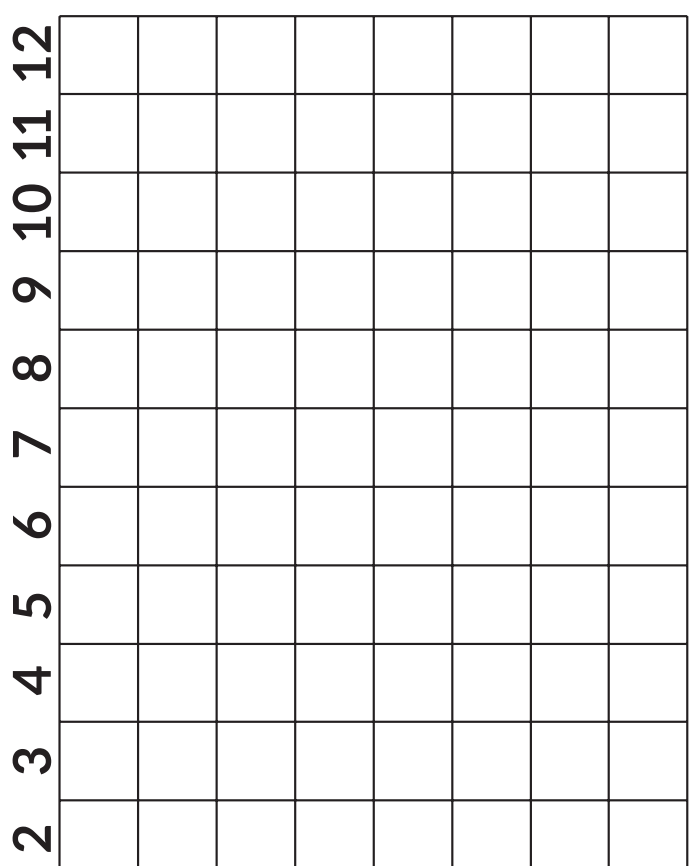
NOTE: The blank plus inhibitor wells should not exhibit an absorbance >0.20. If the absorbance is above 0.20, then try diluting with 1X Assay Buffer or solvent the inhibitor is dissolved in. The H_2O_2 wells and the H_2O_2 plus inhibitor wells should exhibit approximately the same absorbance. If the 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 1X Assay Buffer or solvent.

Troubleshooting

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 (on page 18) 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 (on page 18)
No inhibition was seen with the inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Gaffney, B.J. *Annu. Rev. Biophys. Biomol. Struct.* **25**, 431-459 (1996).
2. Yamamoto, S. *Biochim. Biophys. Acta* **1128**, 117-131 (1992).
3. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. *J. Biomol. Screen.* **4(2)**, 67-73 (1999).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.