



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 ml	4°C
760711	Developing Reagent 1	1 vial/6 ml	4°C
760712	Developing Reagent 2	1 vial/6 ml	4°C
760714	15-Lipoxygenase Enzyme	1 vial/120 µl	4°C
760715	Arachidonic Acid (substrate)	1 vial/300 µl	-20°C
760716	Linoleic Acid (substrate)	1 vial/300 µl	-20°C
760713	Potassium Hydroxide	1 vial/500 µl	4°C
760717	Nordihydroguaiaretic Acid (NDGA) Control Inhibitor	1 vial/ 550 nmol	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate	RT
400012	96-Well Cover Sheet	1 ea	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 at 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. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. An orbital microplate shaker
5. Methanol or DMSO to resuspend the inhibitor
6. Materials used for Sample Preparation (see page 7)

INTRODUCTION

Background

Lipoxygenases, commonly abbreviated as LOs and LOXs, are non-heme iron-containing dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) containing a *cis,cis*-1,4-pentadiene system to form a lipid hydroperoxide.^{1,2} Linoleic acid and arachidonic acid are the predominant substrates for lipoxygenases in plants and mammals, respectively.^{3,4} In humans, there are six LOs that are categorized by the position of oxygenation with arachidonic acid as the substrate: 5-LO, 12R-LO, 12-LO, 15-LO-1, and 15-LO-2, as well as the hydroperoxide isomerase epidermis-type lipoxygenase 3 (eLOX-3).^{5,6} 5-LO localizes to leukocytes, mast cells, dendritic cells, and B lymphocytes, 12R-LO and eLOX-3 to the epidermis, 12-LO to platelets and leukocytes, 15-LO-1 to eosinophils, reticulocytes, and epithelial cells, and 15-LO-2 to the hair follicles, epidermis, and prostate.¹ Arachidonic acid is the primary substrate for LOs, but they also act on linoleic acid, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), α -linolenic acid (ALA), and γ -linolenic acid (GLA). The resulting metabolites are further metabolized to various oxylipins with many being members of several lipid mediator classes such as leukotrienes, lipoxins, hepxilins, eoxins, resolvins, and protectins.^{1,2,7} LOs are implicated in several diseases, including atherosclerosis, asthma, neurodegenerative diseases, diabetes, obesity, and cancer, either directly or *via* the action of their metabolites.^{1,8} The discovery of novel, potent LO inhibitors will aid in understanding the role of LOs in these disease states.⁸

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.

Provided in this kit is arachidonate 15-LO purified from soybean, which can be used to screen test compounds. Alternatively, if other purified LOs (provided by the user) are to be used for screening, the included enzyme can be used as a control. Both arachidonic and linoleic acids are included as substrates. The preferred substrate for screening will depend on the LO being tested. It is recommended to use the fatty acid substrate best suited for the LO being tested.

PRE-ASSAY PREPARATION

Sample Preparation

Test Compounds

Test compounds can be dissolved in DMSO or methanol. Dimethyl formamide (DMF) is not compatible with this assay and ethanol is not recommended to prepare the test compounds. Additional solvents have not been tested. The concentrated compound stock solutions must be further diluted in 1X Assay Buffer to a concentration 11X the desired final assay (*i.e.* in-well) concentration. Appropriate vehicle control wells containing the same concentration of solvent used for the test compounds must be included in each assay.

Reagent Preparation

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

Dilute 3 ml of Assay Buffer concentrate with 27 ml of pure water. When stored at 4°C, this 1X Assay Buffer is stable for at least two months.

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

This reagent is ready to use as supplied.

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

This reagent is ready to use as supplied.

4. 15-Lipoxygenase Enzyme - (Item No. 760714)

This vial contains purified arachidonate 15-LO from soybean.

To screen a full plate of test compounds, prepare a 1:100 dilution by adding 110 µl of arachidonate 15-LO to 10.89 ml of 1X Assay Buffer.

To use as a control, prepare a 1:100 dilution by adding 10 µl of arachidonate 15-LO to 990 µl of 1X Assay Buffer.

The diluted arachidonate 15-LO is stable for one hour on ice.

5. Potassium Hydroxide - (Item No. 760713)

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

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. *NOTE: Arachidonic acid is recommended for mammalian LO reactions and arachidonate 15-LO from soybean.*

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 pure water to achieve a working concentration of 1 mM. Use within 30 minutes. Addition of 10 µl to the well yields a reaction concentration of 91 µM.

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. *NOTE: Linoleic acid is recommended for plant LO reactions.*

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 pure water to achieve a working concentration of 1 mM. Use within 30 minutes. Addition of 10 µl to the well yields a reaction concentration of 91 µM.

8. NDGA Control Inhibitor - (Item No. 760717)

This vial contains the non-selective LO 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.

9. 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 120 µl for each well. Use the prepared Chromogen within one hour.

Enzyme Preparation

NOTE: Read this section carefully if screening test compounds with an enzyme other than the supplied 15-LO.

It is recommended to use highly purified LOs—either recombinant or obtained from natural sources—such as 5-, 12-, or 15-LO (see Table 1 on page 11 for recommendations). Phosphates, EDTA, transition metal ions, thiols, and endogenous LO inhibitors must be removed prior to use. This can be achieved through extensive dialysis or by buffer exchange *via* centrifugal concentrators.

It is not recommended to use crude cell lysates and tissue homogenates with this assay as they may contain peroxidases (*e.g.*, glutathione peroxidase) that will reduce the lipid hydroperoxides generated in the assay, resulting in a very low signal.

Cyclooxygenases should not be measured by this assay. Indomethacin (Item No. 70270) or other non-selective COX inhibitors can be added to the assay to eliminate COX activity.

If using an LO other than the provided soybean 15-LO, first determine its optimal concentration by assaying a range of enzyme concentrations with the NDGA inhibitor and the corresponding vehicle solution. The optimal concentration is the lowest concentration that produces a difference in absorbance between the inhibitor-containing and vehicle control wells of 0.2 to 0.3 and shows at least 90% inhibition with NDGA.

Lipoxygenase	Cayman Item No.	Recommendation	Substrate
5-Lipoxygenase (human, recombinant)	60402	Not suitable for use with this assay	N/A
12-Lipoxygenase (mouse, recombinant)	10341	✓	Arachidonic Acid
15-Lipoxygenase-2 (human, recombinant)	10011263	✓	Arachidonic Acid
Arachidonate 5-Lipoxygenase (potato) Screening Enzyme	60401	✓	Arachidonic Acid or Linoleic Acid

Table 1. Fatty acid substrate preferences of different LO isozymes

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to include blank, vehicle control, and inhibitor control wells in at least duplicate. If using an LO other than the provided 15-LO, it is recommended to include at least two wells for the 15-LO control reaction. A typical layout of samples to be measured in duplicate is shown in Figure 1. It is suggested to record the contents of each well on the template sheet provided (see page 25).

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	6	6	14	14	22	22	30	30	38	38
B	VC	VC	7	7	15	15	23	23	31	31	39	39
C	IC	IC	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	EC	EC

BW = Blank Wells

VC = Vehicle Control Wells

IC = Inhibitor Control Wells

1-44 = Test Compound Wells

EC = 15-LO Control Wells (Optional)

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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 μ l in all the wells.
- It is not necessary to use all the wells on the plate at one time.
- All reagents should be prepared as described above. The enzyme(s) should be kept on ice and all other reagents should be kept at room temperature prior to beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- The assay is performed at room temperature.
- Read the absorbance at 490-500 nm.

Performing the Assay

1. Add the appropriate amount of reagents to the designated wells according to Table 2, below. Add the enzyme last. Thoroughly mix by pipetting up and down, ensuring no bubbles are formed.

	Blank Wells	Vehicle Control Wells	Inhibitor Control Wells	Test Compound Wells	15-LO Control Wells (Optional)
1X Assay Buffer	100 μ l	--	--	--	10 μ l
Solvent	--	10 μ l	--	--	--
NDGA, 1.1 mM	--	--	10 μ l	--	--
Test Compound, 11X	--	--	--	10 μ l	--
Diluted 15-LO Enzyme	--	--	--	--	90 μ l
Optimized lipoxygenase*	--	90 μ l	90 μ l	90 μ l	--

Table 2. Pipetting summary

*User-supplied; see *Enzyme Preparation* on pages 10-11

2. Incubate for five minutes at room temperature.
3. Initiate the reaction by adding 10 μ l of substrate solution (either arachidonic or linoleic acid) to all wells. Incubate on an orbital microplate shaker for ten minutes at room temperature.
4. Add 100 μ l of Chromogen to each well to stop and develop the reaction.
5. Cover the plate with the 96-Well Cover Sheet (Item No. 400012) and shake for an additional five minutes at room temperature.
6. Remove the cover and read the absorbance at 490-500 nm.

ANALYSIS

Calculations

1. Determine the average absorbance for each set of replicates.
2. Subtract the average absorbance of the blank wells from the average absorbance of the 15-LO control, vehicle control, inhibitor control, and test compound wells. These are the corrected values.
3. Using the corrected values, determine the percent inhibition or percent activity for each test compound using one of the following equations:

$$\% \text{ inhibition} = \left[1 - \frac{\text{corrected value of test compound}}{\text{corrected value of vehicle}} \right] \times 100$$

$$\% \text{ activity} = \frac{\text{corrected value of test compound}}{\text{corrected value of vehicle}} \times 100$$

4. Graph the Percent Inhibition or Percent Activity as a function of the test compound to determine the IC_{50} value (concentration at which there was 50% inhibition). Inhibition of soybean 15-LO by NDGA is shown in Figure 2 (see page 17).

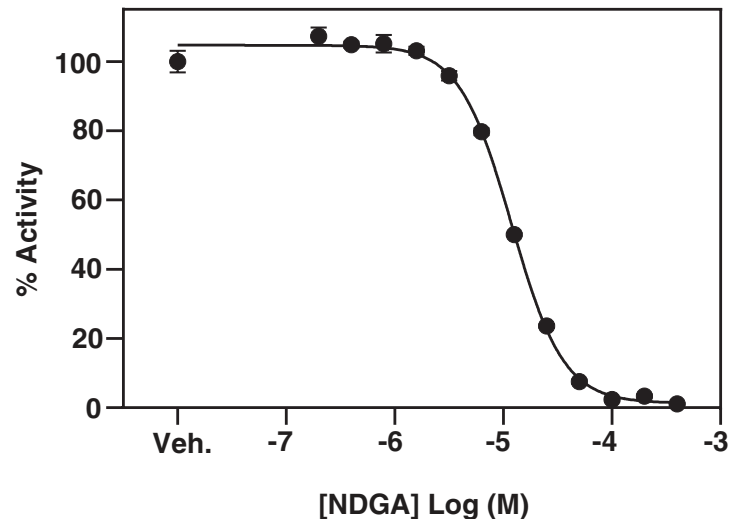


Figure 2. Inhibition of soybean 15-LO by NDGA ($IC_{50} = 12 \mu\text{M}$). Arachidonic acid was used as the substrate in this experiment.

Performance Characteristics

Z' Factor:

Z' factor is a term used to describe the robustness of an assay,⁹ 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 Lipoygenase Inhibitor Screening Assay Kit was determined to be 0.88.

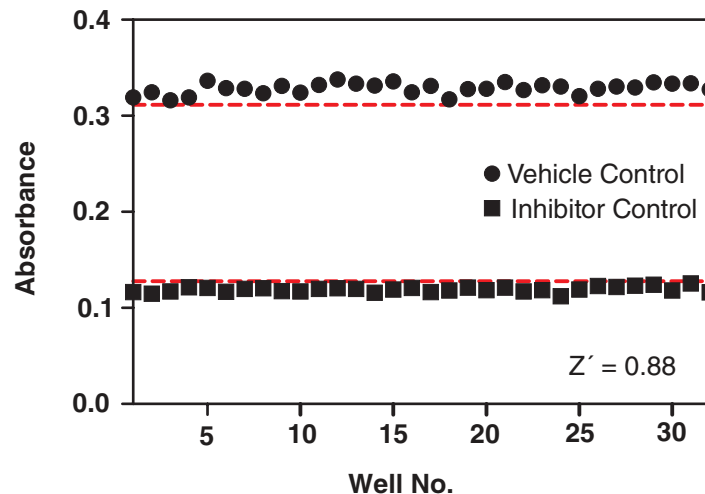


Figure 3. Typical Z' data for the Lipoygenase Inhibitor Screening Assay Kit. Data are shown from wells of both vehicle and inhibitor control wells prepared as described in the kit booklet. Arachidonic acid and the soybean 15-LO enzyme were used as the substrate and LO, respectively. The calculated Z' factor from this experiment was 0.88. The red dashed lines correspond to three standard deviations from the mean for each control value.

RESOURCES

Interferences

Reagents	Will Interfere	
Buffers	Tris	No
	Borate	No
	EGTA	No
	Phosphate	Yes
	HEPES	Yes
	EDTA	Yes
Culture Medium	F-12	No
	MEM	Yes
	DMEM	Yes
Thiols and Transition Metal Ions*	Glutathione	Yes
	Cysteine	Yes
	DTT	Yes
	2-Mercaptoethanol	Yes
	Iron	Yes
	Manganese	Yes
	Copper	Yes
	Solvents	Methanol
DMSO		Slightly
Ethanol		Yes
DMF		Yes

*Extensive dialysis will eliminate most of the interfering substances of small molecular size.

Buffers and culture medium should be tested for high blank absorbance before embarking on a large number of samples. If the initial blank absorbance is higher than 0.22 absorbance units, the buffer or medium should be diluted with 1X Assay Buffer or pure water before performing the assay.

Testing for Interference

NOTE: A 420 μM solution of hydrogen peroxide (H_2O_2) is required for this protocol and will need to be supplied by the user.

It is possible that a test compound for LO inhibition will interfere with the development of the assay. Potential interference can be tested by assaying the test compound in question according to the procedure outlined below.

Add the appropriate amount of prepared reagents to the corresponding wells according to the table below:

Reagent	Blank	Blank + Test Compound	H_2O_2	H_2O_2 + Test Compound
1X Assay Buffer	100 μl	90 μl	90 μl	80 μl
H_2O_2	--	--	10 μl	10 μl
Test Compound	--	10 μl	--	10 μl

1. Initiate the reaction by adding 10 μl of the substrate solution (either arachidonic acid or linoleic acid) to all the wells.
2. Incubate at room temperature on an orbital shaker for ten minutes.
3. Add 100 μl of Chromogen to all the wells.
4. Cover the plate with the 96-Well Cover Sheet and incubate at room temperature on an orbital shaker for five minutes.
5. Remove the plate cover and read the absorbance at 490-500 nm.

Increased absorbance due to the test compound

If the Blank + Test Compound wells exhibit an absorbance greater than 0.2, dilute the test compound with 1X Assay Buffer or solvent and test again.

Calculating the percent interference

1. Determine the average absorbance for each sample.
2. Determine the percent interference of the test compound using the equation below. The percentage interference should be less than 10% to indicate no effect on the assay.

$$\% \text{ Interference} = 100 \times \left(\frac{\text{absorbance of H}_2\text{O}_2 - \text{absorbance of H}_2\text{O}_2 + \text{test compound}}{\text{absorbance of H}_2\text{O}_2} \right)$$

3. If interference is observed, dilute the test compound with 1X Assay Buffer or solvent and test again.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	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 detectable signal in test compound wells	A. Test compound is an LO inhibitor B. Test compound interferes with detection chemistry	A. Confirm inhibitory activity by testing at multiple concentrations to establish dose-dependence B. Follow the protocol on pages 21-22 to determine if the test compound interferes with the color development
High blank absorbance (>0.2)	Test compound interferes with detection chemistry (e.g., reacts with chromogen or absorbs at detection wavelength)	Follow the protocols on pages 21-22 to assess whether the test compound contributes to background signal or interferes with the detection chemistry
Inhibition was not observed in inhibitor control wells	A. Inhibitor control was not added B. The LO concentration is too high	A. Confirm that the inhibitor was added at the correct concentration and dilution B. Optimize the LO concentration (see page 11)
No color development above blank in vehicle control wells	A. Omission of key reagent(s) B. Incorrect substrate used for the LO isozyme being assayed C. LO activity is too low D. Interferants remain in the enzyme preparation	A. Confirm all reagents were added to wells B. Confirm substrate for LO isozyme being used C. Optimize the LO concentration (see page 11) D. Use purified LO prepared in a compatible buffer (see page 10)

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Warranty and Limitation of Remedy

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