CREATIVE BIOMART®
Assay Kit

Lipoxygenase Inhibitor Screening Assay Kit

Product Information

Cat.No. Kit-0519

Product Overview

Lipoxygenase Inhibitor Screening Assay Kit detects and measures the hydroperoxides produced in the lipoxygenation reaction using a purified Lipoxygenases (LOs). 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.

Applications

Functional Studies

Storage

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below
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
Nordihydroguaiaretic Acid (NDGA) Positive Control Inhibitor -20°C
96-Well Solid Plate (Colorimetric Assay) RT
96-Well Cover Sheet RT

Size

1 x 96 well plate

Kit Components

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Components 96 tests
15-Lipoxygenase Standard 1 vial
96-Well Plate (Colorimetric Assay) 1 plate
96-Well Plate Cover 1 cover
Arachidonic Acid (substrate) 1 vial
Developing Reagent 1 1 vial
Developing Reagent 2 1 vial
Linoleic Acid (substrate) 1 vial
Lipoxygenase Inhibitor Screening Assay Buffer (10X) 1 vial
Potassium Hydroxide 1 vial
Nordihydroguaiaretic Acid (NDGA) Positive Control Inhibitor 1 vial

Materials Required 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

Preparation

Reagent Preparation
1. Lipoxygenase Inhibitor Screening Assay Buffer (10X)
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
The reagent is ready to use as supplied.
3. Developing Reagent 2
The reagent is ready to use as supplied.
4. Chromogen
Prepare the Chromogen prior to use by mixing equal volumes of Developing Reagent 1 and

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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.
5. 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 1X Assay Buffer prior to use, store on ice, and use within one hour.
6. 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 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)
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 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
This vial contains 0.1 M potassium hydroxide (KOH). The reagent is ready to use as supplied.
9. NDGA Positive Control Inhibitor
This vial contains 550 nmol of the non-selective lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA). Resuspend in 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.

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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 as a control.

Assay Protocol

A. 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.

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

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• 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. B.

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 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 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.
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

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.

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3. Determine the percent inhibition or percent IA for each inhibitor using one of the following equations.
% Inhibition = (IA - Inhibitor)/ [IA] x 100
% IA = Inhibitor/ IA x 100
4. Graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC50 value (concentration at which there was 50% inhibition).

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.