ab133091 – PAF Acetylhydrolase Inhibitor Screening Assay Kit

Instructions for Use

For screening PAF Acetylhydrolase inhibitors.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

ab133091 uses 2-thio PAF as a substrate for PAF Acetylhydrolase (PAF-AH). Upon hydrolysis of the acetyl thioester bond at the *sn*-2 position by PAF Acetylhydrolase, free thiols are detected using 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB; Ellman's reagent; Figure 1). The PAF Acetylhydrolase Inhibitor Screening assay includes Human plasma PAF Acetylhydrolase and is a time saving tool for screening vast numbers of inhibitors.

2. Background

Platelet-activating factor (PAF) is a biologically active phospholipid synthesized by a variety of cells upon stimulation. The biological effects of PAF include activation of platelets, polymorphonuclear leukocytes, monocytes, and macrophages. PAF also increases vascular permeability, decreases cardiac output, induces hypotension, and stimulates uterine contraction. PAF has been implicated in pathological processes, such as inflammation and allergy. PAF is converted to the biologically inactive lyso-PAF by the enzyme PAF Acetylhydrolase. PAF Acetylhydrolases are located intra- and extra-cellularly (e.g., cytosolic and plasma). Plasma PAF Acetylhydrolase is highly selective for phospholipids with very short acyl groups at the sn-2 position and is associated with lipoproteins. Recently, plasma PAF Acetylhydrolase has been linked to atherosclerosis and may be a positive risk factor for coronary heart disease in Humans.

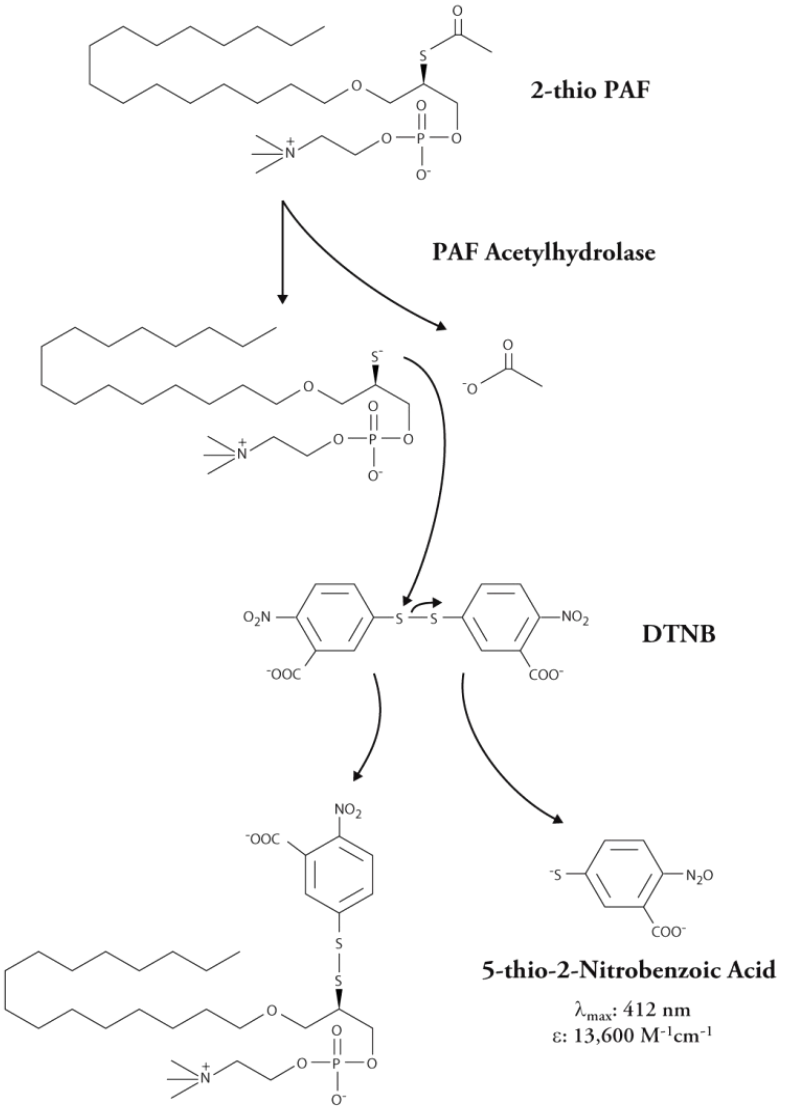

Figure 1. Assay scheme.

3. Components and Storage

This kit will perform as specified if stored at -20°C.

Item	Quantity
PAF-AH Assay Buffer (10X)	1 vial
PAF-AH Assay DTNB	4 vials
2-thio PAF (substrate)	2 vials
Human Plasma PAF-AH	2 vials
96-Well Solid Plate (colorimetric assay)	1
96-Well Cover Sheet	1

Materials Needed But Not Supplied

- A plate reader capable of measuring absorbance between 405-414 nm.
- Adjustable pipettes and a repeat pipettor.
- A source of pure water; glass distilled water or HPLC-grade water is acceptable.

4. Pre-Assay Preparation

Reagent Preparation:

All the kit components are supplied in lyophilized or concentrated form (except the plasma PAF-AH) and need to be reconstituted or diluted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used to prepare the components.

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 7.2) should be used for reconstitution of Substrate and dilution of water-soluble inhibitors. When stored at 4°C, this diluted Assay Buffer is stable for at least six months.

DTNB

Reconstitute the contents of one of the vials with 1.0 ml of HPLC-grade water. Store the reconstituted reagent on ice in the dark and use within eight hours.

2-thio PAF (substrate)

Evaporate the ethanolic solution of 2-thio PAF to dryness under a gentle stream of nitrogen. Reconstitute the contents of each vial by vortexing with 12 ml of diluted Assay Buffer to achieve a concentration of 400 µM. Make sure to vortex until the Substrate

Solution becomes clear. The reconstituted Substrate is stable for two weeks at -20°C. *NOTE: If not using the entire plate, then reconstitute only one of the Substrate vials. The final concentration of 2-thio PAF in the assay as described below is 348 µM. This concentration may be reduced with Assay Buffer at the users discretion, particularly when complete inhibition curves are required for IC_{50} or Ki determination. For competitive inhibitors, the IC_{50} is dependent upon the Substrate concentration and must be reported in the results. An example is exhibited in Figure 3 using the inhibitor methyl arachidonyl fluorophosphonate.*

Human Plasma PAF-AH

These vials contain a solution of Human plasma PAF-AH and should be kept on ice when thawed. The enzyme is ready to use as supplied. *NOTE: If not using the entire plate, then thaw only one of the enzyme vials.*

Sample (inhibitors)

Sample (inhibitors) can be dissolved in methanol, dimethylsulfoxide, or ethanol and should be added to the assay in a final volume of 10 µl. In the event that the appropriate concentration of inhibitor needed for PAF Acetylhydrolase inhibition is completely unknown, we recommend that several concentrations of the inhibitor be tested.

5. Assay Protocol

A. Plate Setup

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% Initial Activity and three wells designated as background wells. A typical layout of PAF Acetylhydrolase samples to be measured in triplicate is given below in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW – Background Wells
A – 100% Initial Activity Wells
1-30 – Inhibitor/Activator Wells

Pipetting Hints:

- It is recommended that an adjustable pipette be used to deliver Substrate, DTNB, and buffer to the wells. This saves time and helps to maintain more precise times of incubation.

- Use different tips to pipette Substrate, DTNB, and sample.

- 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 is 230 µl in all of the wells.

- If the appropriate inhibitor dilution is not known, it may be necessary to assay at several dilutions.

- We recommend assaying samples in triplicate, but it is the user's discretion to do so.

- 30 inhibitor samples can be assayed in triplicate or 45 in duplicate.

B. Performing the Assay

1. 100% Initial Activity Wells - add 200 µl of the 2-thio PAF Substrate Solution and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells. The 100% initial activity wells should exhibit an absorbance of ~0.5.
2. Sample (inhibitor) Wells - add 200 µl of the 2-thio PAF Substrate Solution and 10 µl of inhibitor to three wells.
3. Background wells - add 10 µl of Assay Buffer, 200 µl of the 2-thio PAF Substrate Solution, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
4. Initiate the reactions by adding 10 µl of PAF-AH to 100% Initial Activity and Inhibitor wells. Do not add PAF-AH to the Background Wells. Carefully shake the microplate for 30 seconds to mix and cover with the plate cover. Incubate for 20 minutes at 25°C.
5. Remove the plate cover. Add 10 µl of DTNB to each well to develop the reaction. Carefully shake the microplate and read the absorbance at 414 (or 405) nm after one minute using a plate reader.

6. Data Analysis

A. Calculations

1. Determine the average absorbance of the background, initial activity, and the inhibitor wells.
2. Subtract the absorbance of the background wells from the absorbance of the 100% initial activity and the inhibitor wells.
3. Determine the percent inhibition for each sample. To do this, subtract each inhibitor sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition.
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). The inhibition of Human plasma PAF-AH by methyl arachidonyl fluorophosphonate (MAFP) is shown as an example.

B. Performance Characteristics

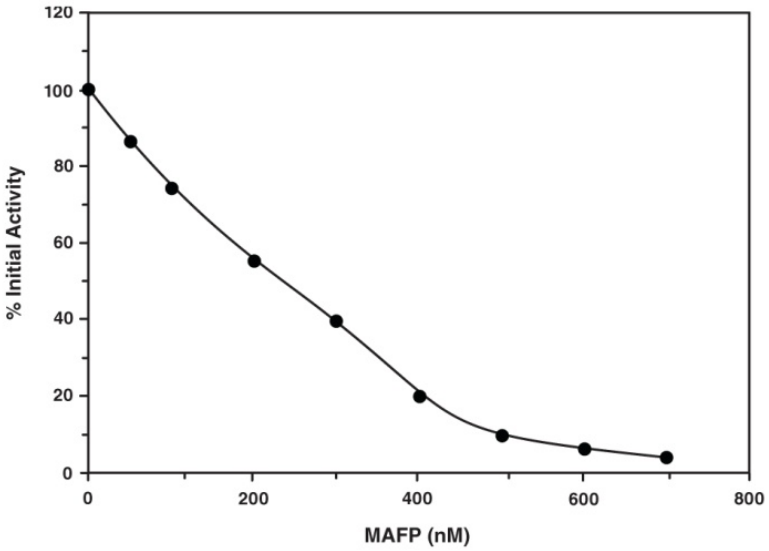

Figure 3. Inhibition of Human plasma PAF-Acetylhydrolase by MAFP (IC_{50} = 250 nM).

C. Interferences

Inhibitors containing thiols will exhibit high absorbance due to the direct reaction with DTNB. Inhibitors that are thiol-scavengers will inhibit color development by preventing the reaction of lyso-thio PAF with DTNB.

7. 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 absorbance above 0.1 is seen in the Inhibitor wells	Enzyme, DTNB, or substrate was not added to the well(s). Inhibitor concentration is too high resulting in complete inhibition of enzyme activity.	Make sure to add all components to the wells. Reduce the concentration of the inhibitor and re-assay.
No inhibition seen with compound	The inhibitor concentration is not high enough or the compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay.

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