PAF Acetylhydrolase Inhibitor Screening Assay Kit

Item No. 10004380

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
10004800	PAF-AH Assay Buffer (10X)	1 vial
10004801	PAF-AH Assay DTNB	4 vials
10004802	2-thio PAF (substrate)	2 vials
10004803	Human Plasma PAF-AH	2 vials
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

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
Fax:	734-971-3641
Email:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 at -20°C 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 405-414 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

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.[1] PAF has been implicated in pathological processes, such as inflammation and allergy.[2] PAF is converted to the biologically inactive lyso-PAF by the enzyme PAF acetylhydrolase (PAF-AH, Lp-PLA_2). PAF-AHs are located intra- and extra-cellularly (*e.g.*, cytosolic and plasma). Plasma PAF-AH is highly selective for phospholipids with very short acyl groups at the *sn*-2 position and is associated with lipoproteins.[3] Recently, plasma PAF-AH has been linked to atherosclerosis and may be a positive risk factor for coronary heart disease in humans.[4]

About This Assay

Cayman's PAF Acetylhydrolase Inhibitor Screening Assay uses 2-thio PAF as a substrate for PAF-AH.[5] Upon hydrolysis of the acetyl thioester bond at the *sn*-2 position by PAF-AH, free thiols are detected using 5,5'-dithio-*bis*-(2-nitrobenzoic acid) also known as DTNB or Ellman's Reagent (Figure 1, see page 7). The PAF Acetylhydrolase Inhibitor Screening Assay includes human plasma PAF-AH and is a time saving tool for screening vast numbers of inhibitors.

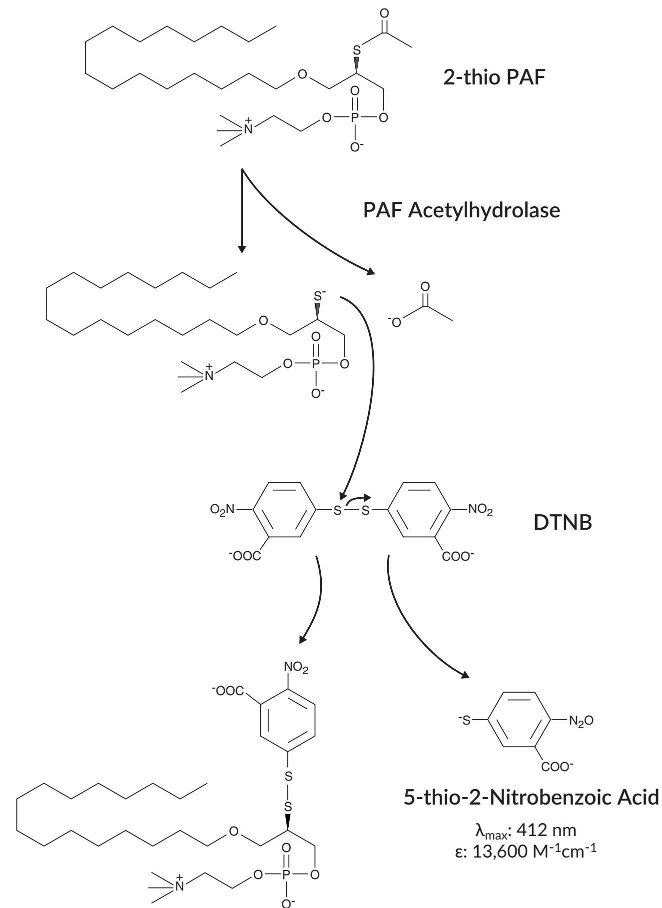

Figure 1. Scheme 1

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.

1. **Assay Buffer (10X) - (Item No. 10004800)**

   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.

2. **DTNB - (Item No. 10004801)**

   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.

3. **2-thio PAF (substrate) - (Item No. 10004802)**

   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 K_i determination. For competitive inhibitors, the IC_{50} value is dependent upon the Substrate concentration and must be reported in the results. An example is exhibited in Figure 3 on page 14 using the inhibitor methyl arachidonyl fluorophosphonate.

4. **Human Plasma PAF-AH - (Item No. 10004803)**

   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.*

ASSAY PROTOCOL

Plate Set Up

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-AH samples to be measured in triplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 18).

	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 Wells

Figure 2. Sample plate format

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.

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. **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.

*Inhibitors can be dissolved in methanol, DMSO, 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-AH inhibition is completely unknown, we recommend that several concentrations of the inhibitor be tested.

ANALYSIS

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 below as an example.[6]

Performance Characteristics

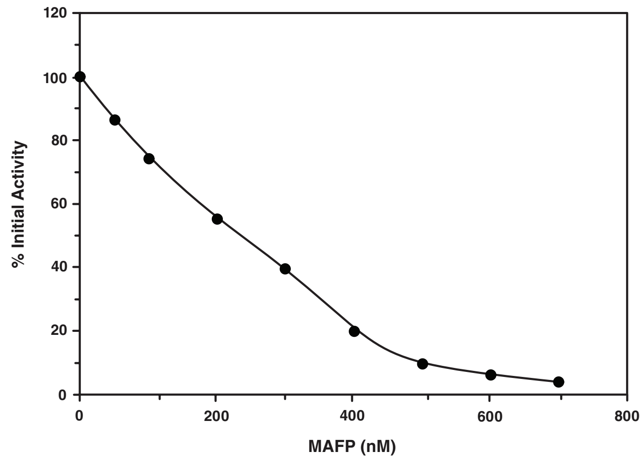

Figure 3. Inhibition of human plasma PAF-AH by MAFP (IC_{50} = 250 nM).

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.

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 inhibitor	The inhibitor concentration is not high enough or the compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. Platelet-activating factor. *J. Biol. Chem.* **265**, 17381-17384 (1990).
2. Snyder, F. Platelet-activating factor and related acetylated lipids as potent biologically active cellular mediators. *Am. J. Physiol. Cell Physiol.* **259**, C697-C708 (1990).
3. Stafforini, D.M., Prescott, S.M., and McIntyre, T.M. Human plasma platelet-activating factor acetylhydrolase. *J. Biol. Chem.* **262**, 4223-4230 (1987).
4. Caslake, M.J. and Packard, C.J. Lipoprotein-associated phospholipase A_2 (platelet-activating factor acetylhydrolase) and cardiovascular disease. *Curr. Opin. Lipidol.* **14**, 347-352 (2003).
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6. Kell, P.J., Creer, M.H., Crown, K.N., *et al.* Inhibition of platelet-activating factor (PAF) acetylhydrolase by methyl arachidonyl fluorophosphonate potentiates PAF synthesis in thrombin-stimulated human coronary artery endothelial cells. *J. Pharmacol. Exp. Ther.* **307(3)**, 1163-1170 (2003).

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								

NOTES

Warranty and Limitation of Remedy

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