ab133088 – PAF Acetylhydrolase Assay Kit

Instructions for Use

For the measurement of PAF-AH activity (both cytosolic and extracellular).

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

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1. Overview

ab133088 provides an accurate and convenient method for measurement of PAF-AH activity (both cytosolic and extracellular). The assay uses 2-thio PAF which serves as a substrate for all PAF-AHs. 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).

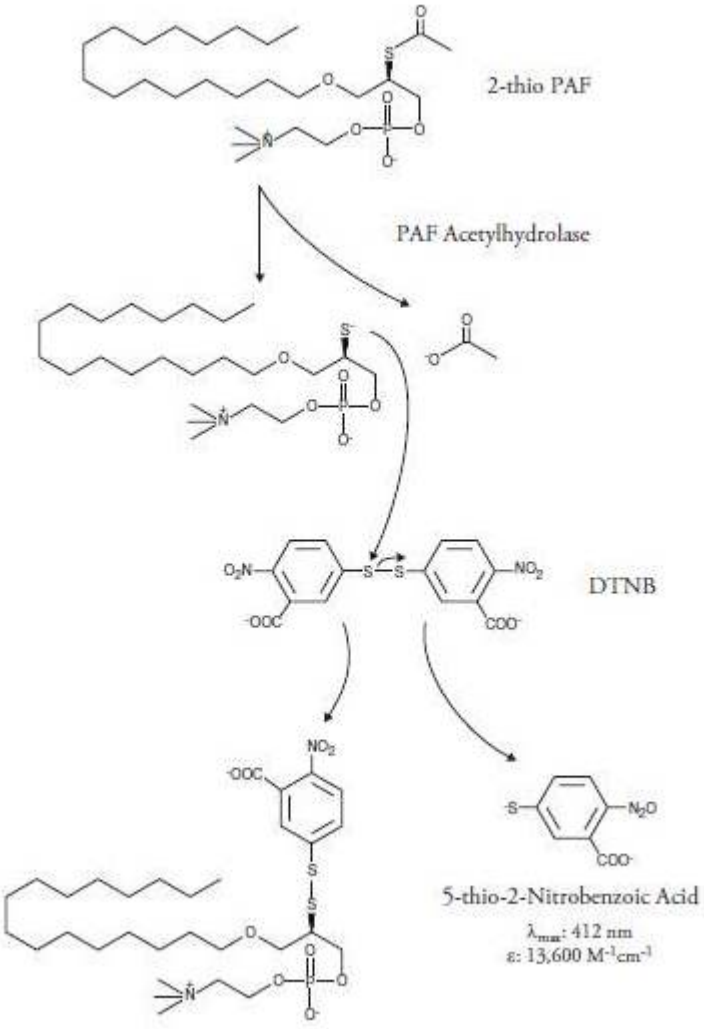

Figure 1. Assay scheme

2. Background

Platelet-activating factor (PAF) is a biologically active phospholipid synthesized by a variety of cells upon stimulation. PAF is converted to the biologically inactive lyso-PAF by the enzyme PAF acetylhydrolase (PAF-AH). 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. Components and Storage

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

Item	Quantity
PAF Acetylhydrolase Assay Buffer 1	1 vial
DTNB	4 vials
2-thio PAF (substrate)	2 vials
Human Plasma PAF-AH Standard	1 vial
PAF Acetylhydrolase Assay Buffer 2	1 vial
96-Well Solid Plate (Colorimetric Assay)	1 plate
96-Well Cover Sheet	1 cover

Materials Needed But Not Supplied

- A plate reader capable of measuring absorbance at 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

A. Reagent preparation

Some of the kit components are in lyophilized form and need to be reconstituted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used to reconstitute the vial components. If assaying for extracellular PAF-AH, use diluted Assay Buffer 1. If assaying for cytosolic PAF-AH, use diluted Assay Buffer 2.

"Sample (inhibitors)" can be dissolved in dimethyl sulfoxide (DMSO), methanol, or ethanol and should be added to the assay in a final volume of 5 µl. In the event that the appropriate concentration of inhibitor is completely unknown, we recommend that several different dilutions of the inhibitor in solvent be made.

PAF Acetylhydrolase Assay Buffer 1 - (Used if assaying for extracellular PAF-AH)

Dilute 3 ml of Assay Buffer 1 concentrate with 27 ml of HPLC-grade water. This final Assay Buffer (0.1 M Tris-HCl, pH 7.2, containing 1 mM EGTA) should be used for reconstitution of substrate and dilution of samples prior to assaying for extracellular PAF-AH.

DTNB

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

2-thio PAF (substrate)

Evaporate the ethanolic solution of 2-thio PAF under a gentle stream of nitrogen. Reconstitute the contents of each vial by vortexing with 12 ml of either diluted Assay Buffer 1 or diluted Assay Buffer 2 to achieve a final concentration of 200 µM. Make sure to vortex until the substrate solution becomes clear (high background absorbance may result if the substrate is not completely dissolved). We recommend using the reconstituted substrate within two weeks.

Human Plasma PAF-AH Standard

A solution of human plasma PAF-AH is supplied as a positive control. A 10 µl aliquot of the enzyme per well causes an increase of approximately 0.025 absorbance unit/min. when assaying for extracellular PAF-AH.

PAF Acetylhydrolase Assay Buffer 2 - (Used if assaying for Cytosolic PAF-AH)

Dilute 3 ml of Assay Buffer 2 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 samples prior to assaying for cytosolic PAF-AH.

B. Sample Preparation

In general, any PAF-AH sample can be measured by this assay. However, cytosolic PAF-AH has to be measured using an end-point assay instead of a continuous assay. Cytosolic PAF-AH is sensitive to DTNB and EGTA. The sample must be free of particulates to avoid interference in the absorbance measurement. Thiols, thiol-scavengers, and PAF-AH inhibitors must be removed from the samples before performing the assay (extensive dialysis will eliminate most of the interfering substances of small molecular size). If the samples are too dilute, they can be concentrated using an Amicon centrifuge concentrator with a molecular weight cut-off of 30,000.

Tissue Homogenate

1. Prior to dissection, rinse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, to remove any red blood cells and clots.

2. Homogenize the tissue in 5-10 ml of cold buffer (i.e., 0.1 M Tris-HCl, pH 7.2) per gram tissue.

3. Centrifuge at 10,000 x g for 15 minutes at 4°C.

4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell lysate

1. Collect cells by centrifugation (i.e., 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.

2. Homogenize or sonicate cell pellet in 1 ml of cold buffer (i.e., 0.1 M Tris-HCl, pH 7.2).

3. Centrifuge at 10,000 x g for 15 minutes at 4°C.

4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Plasma

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.

2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.

   Plasma contains PAF-AH, however, the concentration of the enzyme is too low to be measured directly. Generally plasma should be concentrated to one-fourth its original volume before assaying. Plasma can be concentrated using an centrifuge concentrator with a molecular weight cut-off of 30,000. Make sure to perform controls without substrate to detect non-enzymatic activity (initiate with 200 µl of Assay Buffer). This activity should be subtracted from the extracellular PAF-AH activity.

   Serum samples:

1. Collect blood without an anticoagulant.

2. Allow blood to clot for 30 minutes at 25°C.

3. Centrifuge at 2,000 x g for 15 minutes at 4°C. Transfer the serum (upper layer) to a clean test tube being careful not to

disrupt the white buffy layer. Store serum on ice until assaying or freeze at -80°C. The serum sample should be stable for at least one week.

5. Assay Protocol

A. Assaying for Extracellular PAF-AH

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 225 µl in all of the wells.*
- *Use diluted Assay Buffer 1, containing EGTA, in the assay.*
- *It is not necessary to use all of the wells on the plate at one time.*
- *If the appropriate enzyme dilution is not known, it may be necessary to assay at several dilutions.*

Performing the assay

1. **Blank Wells (no-enzyme controls) -** add 10 µl DTNB and 15 µl Assay Buffer to at least two wells (if performing "sample (inhibitor)" studies, add 5 µl solvent and 10 µl Assay Buffer instead of 15 µl Assay Buffer).

2. **Positive Control Wells (Human PAF-AH) -** add 10 µl DTNB, 10 µl PAF-AH, and 5 µl Assay Buffer to at least two wells (if performing "sample (inhibitor)" studies, add 5 µl solvent instead of 5 µl Assay Buffer)

3. **Sample Wells** - add 10 µl DTNB, 10 µl sample, and 5 µl Assay Buffer to at least three wells (if performing " sample (inhibitor)" studies, add 5 µl of "sample (inhibitor)" dissolved in solvent instead of 5 µl Assay Buffer). To obtain reproducible results, the amount of PAF-AH added to the well should cause an absorbance increase between 0.01 and 0.1/min. When necessary, samples should be concentrated or diluted with Assay Buffer to bring the enzyme activity to this level. NOTE: The amount of sample added to the well should always be 10 µl.

4. Initiate the reactions by adding 200 µl substrate solution to all of the wells. Make sure to note the precise time you started and add the substrate solution as quickly as possible.

5. Carefully shake the 96-well plate for 30 seconds to mix.

6. Read the absorbance once every minute at 405-414 nm using a plate reader to obtain at least five time points

B. Assaying for Cytosolic PAF-AH

General Information

- *The final volume is 225 µl in all of the wells.*
- *Use diluted Assay Buffer 2 in the assay.*
- *It is not necessary to use all of the wells on the plate at one time.*
- *If the appropriate enzyme dilution is not known, it may be necessary to assay at several dilutions.*

Performing the Assay

1. **Blank Wells (no-enzyme controls)** - add 15 µl Assay Buffer to at least two wells (if performing "sample (inhibitor)" studies, add 5 µl solvent and 10 µl Assay Buffer instead of 15 µl Assay Buffer).

2. **Positive Control Wells (Human PAF-AH)** - add 10 µl PAF-AH and 5 µl Assay Buffer to at least two wells (if performing "sample (inhibitor)" studies, add 5 µl solvent instead of 5 µl Assay Buffer). The positive control will yield an absorbance of approximately 0.67 when incubated for 30 minutes.

3. **Sample Wells** - add 10 µl sample, and 5 µl Assay Buffer to at least three wells (if performing “sample (inhibitor)” studies, add 5 µl of “sample (inhibitor)” dissolved in solvent instead of 5 µl Assay Buffer). To obtain reproducible results, the amount of PAF-AH added to the well should result in an absorbance between 0.2 and 1.2 or is at least 2-fold higher than the background absorbance. When necessary, samples should be concentrated or diluted with Assay Buffer to bring the enzyme activity to this level. NOTE: The amount of sample added to the well should always be 10 µl.

4. Initiate the reactions by adding 200 µl substrate solution to all the wells. Carefully shake the 96-well plate for 30 seconds to mix and then cover with plate cover. Incubate for 30 minutes at room temperature.

5. Remove the plate cover. Add 10 µl of DTNB to each well to develop the reaction. Carefully shake the 96-well plate and read the absorbance at 405-414 nm after one minute using a plate reader.

6. Data Analysis

A. Calculations

Determination of reaction rate for extracellular PAF-AH

1. Determine change in absorbance (ΔA414) per minute by:

   a. Plotting the average values as a function of time to obtain the slope (rate) of the linear portion of the curve (a graph is shown using 20 ng of human PAF-AH.

   or

   b. Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{414}/\text{min.} = \frac{A_{414}\ (\text{Time 2}) - A_{414}\ (\text{Time 1})}{\text{Time 2 (min.)} - \text{Time 1 (min.)}}$$

2. Determine the rate of ΔA_{414}/min. for the no-enzyme controls (Blanks) and subtract this rate from that of the sample wells.

3. Use the following formula to calculate the PAF-AH activity. The reaction rate at 414 nm can be determined using the DTNB extinction coefficient of 10.66 mM (The actual extinction coefficient for DTNB at 412 nm is 13.6 $mM^{-1}cm^{-1.}$

This value has been adjusted for the pathlength of the solution in the well (0.784 cm). The extinction coefficient for DTNB at 405 nm is 12.8 $mM^{-1}cm^{-1}$ and the adjusted value would be 10.0 mM^{-1}). One unit of enzyme hydrolyzes one µmol of 2-thio PAF per minute at 25°C.

$$\text{PAF-AH Activity} = \frac{\Delta A_{414}/\text{min.}}{10.66\ mM^{-1}} \times \frac{0.225\ ml}{0.01\ ml} \times \frac{\text{Sample}}{\text{dilution}} = \mu mol/min/ml$$

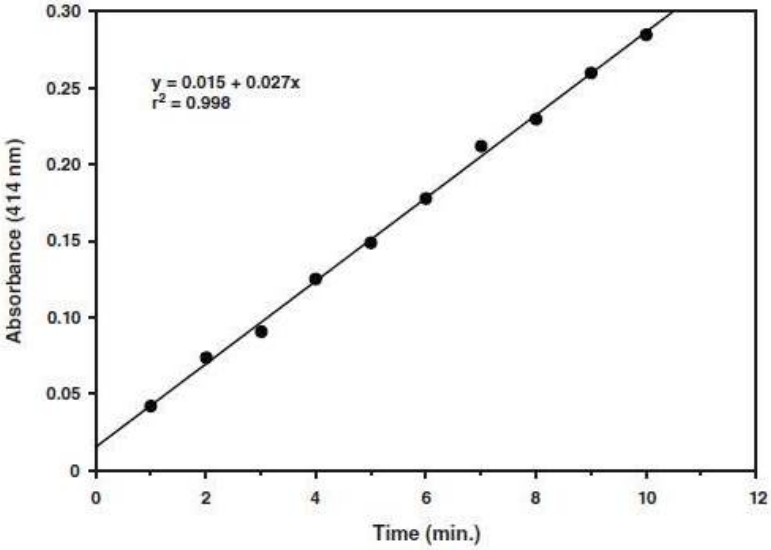

Figure 2: Activity of human PAF-AH

Determination of cytosolic PAF-AH activity

1. Subtract the absorbance of the no-enzyme controls (Blanks) from the absorbance of the samples and divide by the length of incubation (30 minutes).

$$\Delta A_{414}/\text{min.} = \frac{A_{414}\ (\text{sample}) - A_{414}\ (\text{blank})}{\text{Time (30 min.)}}$$

2. Use the following formula to calculate the PAF-AH activity. The reaction rate at 414 nm can be determined using the DTNB extinction coefficient of 10.66 mM (The actual extinction coefficient for DTNB at 412 nm is 13.6 $mM^{-1}cm^{-1}$. This value has been adjusted for the pathlength of the solution in the well (0.784 cm). The extinction coefficient for DTNB at 405 nm is 12.8 $mM^{-1}cm^{-1}$ and the adjusted value would be10.0 mM^{-1}). One unit of enzyme hydrolyzes one µmol of 2-thio PAF per minute at 25°C.

$$\text{PAF-AH Activity} = \frac{\Delta A_{414}/\text{min.}}{10.66\ mM^{-1}} \times \frac{0.225\ \text{ml}}{0.01\ \text{ml}} \times \text{Sample dilution} = \mu\text{mol/min/ml}$$

B. Performance characteristics

Sensitivity:

The detection range of the assay is from 0.02 to 0.2 µmol/min/ml of PAF acetylhydrolase activity which is equivalent to an absorbance increase of 0.01 to 0.1 per minute.

Precision:

When a series of 89 PAF-AH measurements were performed on the same day, the intraassay coefficient of variation was 3.5%. When a series of 89 PAF-AH measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 10%.

C. Interferences

Solvents

Methanol, ethanol, and DMSO have no effect on PAF-AH activity. PAF-AH inhibitors can be dissolved in any of the above solvents and only 5 µl added to the assay.

Culture Media and Buffers

All buffers and media should be tested for high background absorbances before doing any experiments. If the initial background absorbances are higher than 0.3 absorbance units then the samples should be diluted in Assay Buffer before performing the assay. Tris, Hepes, and phosphate buffers work in the assay.

Thiols and Thiol-Scavengers

Samples containing thiols (i.e., glutathione, cysteine, dithiothreitol, or 2-mercaptoethanol) will exhibit high background absorbances and interfere with PAF-AH activity determination. Samples containing thiol-scavengers (i.e. N-ethylmaleimide) will inhibit color development. Extensive dialysis will eliminate most of the interference substances of small molecular size.

7. Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/ triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Carefully tap the side of the plate with your finger to remove bubbles B. Be careful not to splash the contents of the wells
No color development	A. DTNB or sample was not added to well(s) B. The enzymatic activity was too low	A. Make sure to add all components to the wells B. Standardize the assay with the human PAFAH standard. C. Concentrate your sample so that the enzyme activity is in the assay's detection range.
The color development was too fast	Too much enzyme added to well(s)	Dilute your samples with diluted Assay Buffer and re-assay
High background absorbance (>0.3)	A. Substrate not in solution B. Thiols present in sample	A. Make sure to vortex the substrate until a clear solution is made B. Remove thiols or thiol reagents from sample

The reaction rate is not linear at high absorbance	Plate reader not sensitive enough at high absorbance	A. Use only the points at lower concentrations in the linear portion for making the curve B. Dilute your sample with diluted Assay Buffer and re-assay

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