PAF Acetylhydrolase Assay Kit

Catalog Number KA1354

96 assays

Version: 03

Intended for research use only

www.abnova.com

Table of Contents

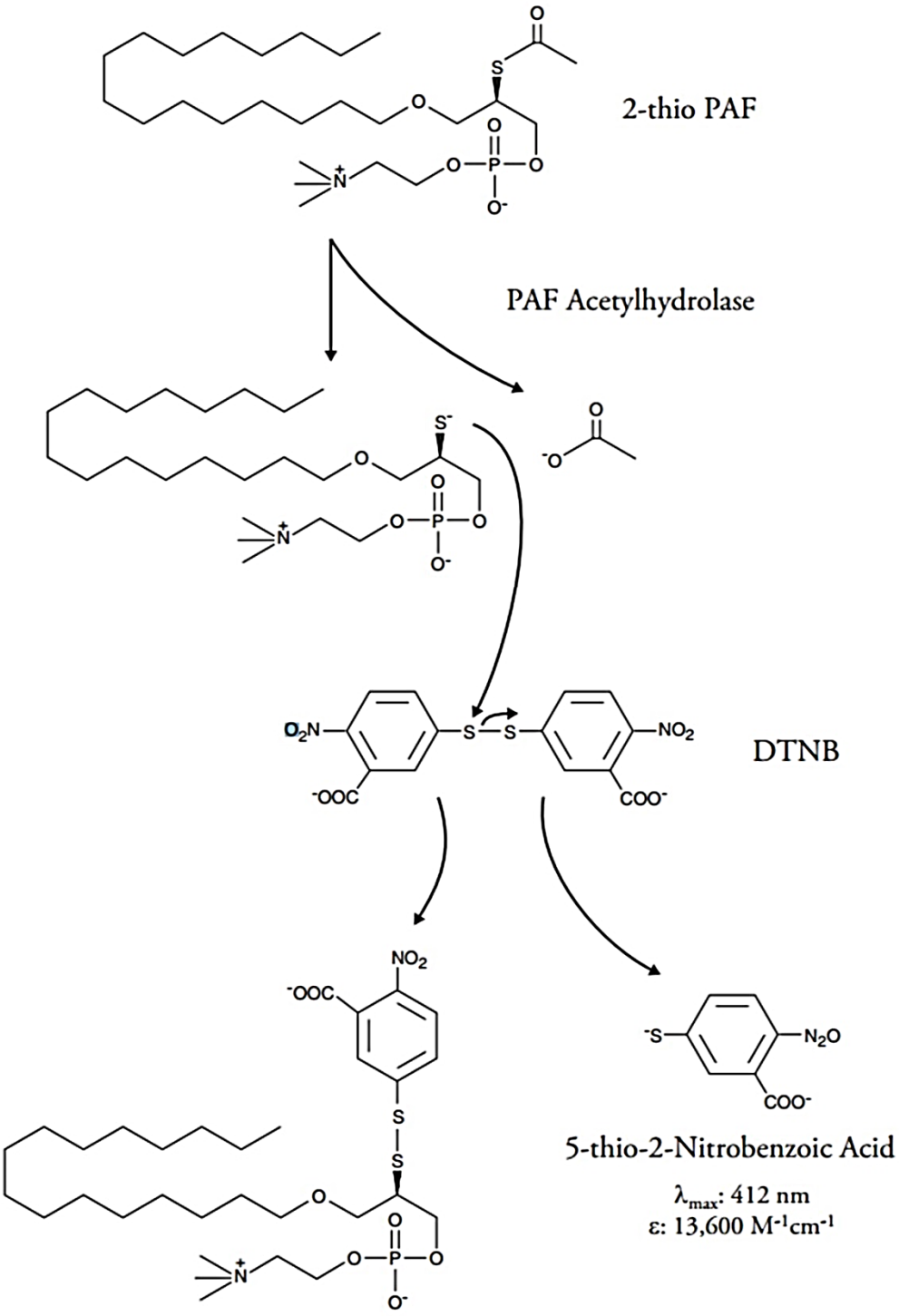
Introduction

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.[1]

Principle of the Assay

PAF Acetylhydrolase Assay Kit 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.[2] 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) (DTNB; Ellman's reagent).


2-thio PAF
PAF Acetylhydrolase
DTNB
5-thio-2-Nitrobenzoic Acid
λmax: 412 nm
ε: 13,600 M-1cm-1

General Information

Materials Supplied

List of component

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

Storage Instruction

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 Required 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

Precautions for Use

Warning: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- ✓ General Information

Assaying for Extracellular PAF-AH

- 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.

Note: Enzymatic reaction rates are temperature dependent. Be diligent about maintaining consistency in temperature with all samples measured. When compareing reaction rates to those reported in the literature, be aware of potential differences in rate based on the temperature used.

Assaying for Cytosolic PAF-AH

- The final volume is 225 µL in all of the wells.
- Use diluted Assay Buffer 2.
- 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.

✓ 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.

✓ Interferences

- Solvent

  Methanol, ethanol, and DMSO have no effect on PAF-AH activity. PAF-AH inhibitors can be dissolved in any of 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 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.

- Culture Media and Buffers

  Samples containing thiols (i.e., glutathione, cysteine, dithiothreitol, or 2-mercaptoethanol) will exhibit high background absorbance 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.

Assay Protocol

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 dilute Assay Buffer 1. If assaying for cytosolic PAF-AH, use dilute Assay Buffer 2.

- ✓ PAF Acetylhydrolase Assay Buffer 1 - 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.

- ✓ PAF Acetylhydrolase Assay Buffer 2 - 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.

- ✓ 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.[3]

- ✓ Human 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.

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.[4] 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. Transfer the plasma (upper layer) to a clean test tube being careful not to disrupt 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 week.

✓ Serum

1. Collect blood without an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood 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 will be stable for at least one week.

Assay Procedure

✓ Assaying for Extracellular PAF-AH

1. No-Enzyme Control Wells - add 10 µL DTNB and 15 µL Assay Buffer to at least two wells (if performing 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 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 inhibitor studies,* add 5 µL of 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. Cover with the plate cover. Incubate for 30 minutes at room temperature to allow any free thiols in the sample to react with DTNB.
5. 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.
6. Carefully shake the 96-well plate for 30 seconds to mix.
7. Read the absorbance once every minute at 405-414 nm using a plate reader to obtain at least five time points. The reaction is linear to at least 1.2 absorbance units.

*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.

Well	Buffer (µL)	PAF-AH (µL)	Sample (µL)	DTNB (µL)	Incubate (30 minutes)	Substrate (µL)
No-Enzme Control	15	--	--	10		200
Positive Control	5	10	--	10		200
Sample	5	--	10	10		200

Table 1. Extracellular PAF-AH pipetting summary

✓ Assaying for Cytosolic PAF-AH

1. No-Enzyme Control Wells - add 15 µL Assay Buffer to at least two wells (if performing 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 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 inhibitor studies, * add 5 µL of 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. Sample Background Wells – add 10 µL sample, and 205 µL Assay Buffer to as least two wells (if performing inhibitor studies, add 5 µL solvent and 200 µL Assay Buffer instead of 205 µL Assay Buffer). Do not add substrate solution to these wells. These wells will correct for any endogenous thiol reactivity in the sample.
5. Initiate the reactions by adding 200 µL substrate solution to all the wells. Carefully shake the 96-well plate for 3*0 seconds to mix and then cover with plate cover. Incubate for 30 minutes at room temperature.*
6. *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.*

   **Inhibitors can be dissolved in 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.*

Well	Buffer (µL)	PAF-AH (µL)	Sample (µL)	DTNB (µL)		Substrate (µL)
No-Enzme Control	15	--	--	200	Incubate (30 minutes)	10
Positive Control	5	10	--	200		10
Sample	5	--	10	200		10
Sample Background	205	--	--	--		10

Table 2. Cytosolic PAF-AH pipetting summary

Data Analysis

Calculation of Results

✓ Determination of reaction rate for extracellular PAF-AH

1. At each time point, determine the average absorbance of the No-Enzyme Control well.
2. Substract these values from all sample values for each respective time point.
3. Determine the change in corrected absorbance (ΔA_{412}) per minute by:

- 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 human recombinant PAF-AH (see Figure 1).
- Select two points on the linear portion of the curve and determine the change in absorbance during that time using the following equation:

$$\Delta A_{412}/\text{min.} = \frac{A_{412}(\text{Time 2}) - A_{412}(\text{Time 1})}{\text{Time 2 (Min.)} - \text{Time 1 (min.)}}$$

- Use the following formula to calculate the PAF-AH activity. The reaction rate at 412 min can be determined using the DTNB extinction coefficient of 10.66 mM. One unit of enzyme hydrolyzes one µmol of 2-thiol PAF per minute at 25°C.

$$\text{PAF - AH Activity } (\mu\text{mol/min/mL}) = \frac{\Delta A_{412}/\text{min}}{10.66\,\text{mM}^{-1}} \text{ x } \frac{0.225\,\text{mL}}{0.01\,\text{mL}} \text{ x Sample dilution}$$

The actual extinction coefficient for DTNB at 412 nm is 13.6 $mM^{-1}cm^{-1}$. This value has been adjusted for the pathlength for the solution in the well (0.784 cm). The extinction coefficient for DTNB at 405 nm is 12.8 $nM^{-1}cm^{-1}$ and the adjusted value would be 10.0 mM^{-1}.

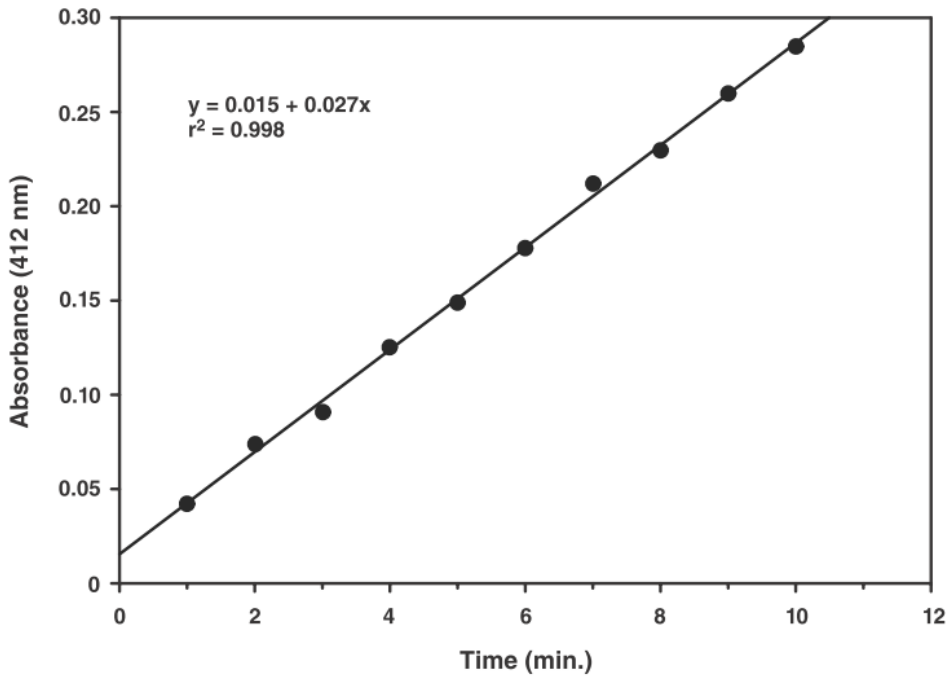


Figure 1. Assay of human recombinant PAF-AH

✓ Determination of reaction rate for extracellular PAF-AH

1. Substrate the absorbance of the No-Enzyme Control wells from all other wells on the plate.
2. Us the following equation to calculate the enzymatic change in the absorbance as a function of time.

$$\Delta A_{412}/\text{min.} = \frac{A_{412}(\text{sample}) - A_{412}(\text{samplebackground})}{\text{Time (30 Min.)}}$$

3. Us the following to calculate the PAF-AH activity. The reaction rate at 412 nm can be determined using the DTNB extinction coefficient of 10.66 mM. One unit of enzyme hydrolyzes one µmol of 2-thiol PAF per minute at 25°C.

$$\text{PAF - AH Activity } (\mu\text{mol/min/mL}) = \frac{\Delta A_{412}/\text{min}}{10.66\,\text{mM}^{-1}} \text{ x } \frac{0.225\,\text{mL}}{0.01\text{mL}} \text{ x Sample dilution}$$

The actual extinction coefficient for DTNB at 412 nm is 13.6 $mM^{-}1cm^{-1}$. This value has been adjusted for the pathlength for the solution in the well (0.784 cm). The extinction coefficient for DTNB at 405 nm is 12.8 $mM^{-}1cm^{-1}$ and the adjusted value would be 10.0 mM^{-1}.

Performance Characteristics

- Sensitivity

  The detection range of the assay is from 0.02 to 0.2 µmol/min/mL of PAE 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, intra-assay 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%.

Resources

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 PAF-AH 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

References

1. Stafforini, D.M., Prescott, S.M., and McIntyre, T.M. Human plasma platelet-activating factor acetylhydrolase. *J. Biol. Chem.* 262, 4223-4230 (1987).
2. Aarsman, A.J., Neys, F.W., and van den Bosch, H. Catabolism of platelet-activating factor and its acyl analog. Differentiation of the activities of lysophospholipase and platelet-activating-factor acetylhydrolase. *Eur. J. Biochem.* 200, 187-193 (1991).
3. Stewart, A.G. and Grigoriadis, G. Structure-activity relationships for platelet-activating factor (PAF) and analogs reveal differences between PAF receptors on platelets and macrophages. *J. Lipid Mediators* 4, 299-308 (1991).
4. Narahara, H., Nishioka, Y., and Johnston, J.M. Secretion of platelet-activating factor acetylhydrolase by human decidual macrophages. *J. Clin. Endocrinol. Metab.* 77, 1258-1262 (1993).

Plate Layout

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