

## 1. INTRODUCTION

PAI-1 is a glycoprotein with an approximate molecular weight of 50 kD. It is a member of the serine protease inhibitor (Serpine) superfamily with homology to anti-thrombin III, PAI-2, alpha-2-antiplasmin, C1-inhibitor, and alpha-1 protease inhibitor. PAI-1 reacts about equally well with single and double chain t-PA and with double chain urokinase, but does not react with single chain urokinase. The interaction between PAI-1 and t-PA is reported to be about 5-10-fold slower in the presence of fibrin. The interaction of the plasminogen activator with PAI-1 probably results first in the formation of a reversible complex which in a second step, becomes covalent after the cleavage of a peptide bond in the PAI-1 molecule. In addition to circulating plasma PAI-1, a large quantity of this glycoprotein is contained in the alpha granules of platelets from which it is released following appropriate stimuli. PAI-1 is also produced by the liver and by endothelial cells. Three forms of PAI-1 have been identified. The active form represents only a small proportion of total circulating PAI-1 (approximately 3.5 - 10.5 t-PA inhibiting units/ml). It is found complexed to the PAI-1 binding protein, vitronectin. A larger amount of PAI-1 circulates in a latent form, which can be converted to the active form by phospholipids. The third form is inactive PAI-1 or t-PA-PAI-1 complexes. This form cannot be converted to the active substance. PAI-1 is an acute phase protein and its plasma concentration increases in conditions where increased Interleukin I levels are observed, e.g. infections, some malignancies and during the postoperative period. Elevated PAI-1 levels are also associated with myocardial infarction and coronary artery disease.

## 2. APPLICATIONS

The Actibind-PAI-1-ELISA can be used to measure active PAI-1 levels in patients with thrombotic disorders (deep vein thrombosis, myocardial infarction, stroke), malignancies or septicemia.

## 3. TEST PRINCIPLE

The TC Actibind-PAI-1 test is a solid phase enzyme immunoassay in which an anti-t-PA monoclonal antibody is utilized to immobilize t-PA onto a plastic microtitre plate. The t-PA active site remains exposed and is used to bind active PAI-1 contained in plasma samples or a standard. An enzyme-labelled (POX= horse radish peroxidase) monoclonal antibody POX-MAb which recognizes a specific site on the PAI-1 molecule is incubated simultaneously with the sample on the plate. The quantity of POX-MAb which binds is proportional to the quantity of active PAI-1 contained in test samples. Unbound POX-MAb is washed away and a substrate which reacts with the peroxidase enzyme is added, leading to a colour change proportional to the amount of enzyme bound. The enzyme reaction is stopped after a specific incubation time. The absorbances of the wells are then measured and the values obtained used to construct a standard curve from which patients' sample values can be extrapolated.

## 4. KIT COMPONENTS

Determinations: 42 samples in duplicate

- **MICROTITRE PLATE**  
12x8 well plastic microtitre strips precoated with t-PA immobilised onto the plate by use of a monoclonal anti-t-PA coating antibody. (TC-Code GP).
- **STANDARD 1 to 5**  
5x lyophilized purified plasma recombinant PAI-1, added to t-PA/PAI-1 depleted plasma, calibrated against NIBSC 92/654 (TC-Code DW 1 to 5) For standard concentrations see last page.
- **CONTROL PLASMA A and B**  
2x lyophilized human plasmas (TC-Code ADW and BDW)
- **POX-ANTIBODY**  
1x conjugated monoclonal anti PAI-1 antibodies (concentrated) blue colour (TC-Code KQ)
- **SAMPLE DILUTION BUFFER - (white cap)**  
1x 20 ml 2.5-fold concentrated PBS with BSA and EDTA. (TC-Code AD)
- **SUBSTRATE – (green cap)**  
1x 12 ml TMB (Tetramethylbenzidine) in substrate buffer containing H<sub>2</sub>O<sub>2</sub>. Ready to use. (TC-Code KN)
- **STOP SOLUTION - (red cap)**  
1x 12 ml 0.5 M Sulphuric Acid (TC-Code KK). Ready to use
- **WASH BUFFER - (blue cap)**  
2x 20 ml 12.5-fold concentrated PBS with Tween 20 (TC-Code BE)

## ALSO REQUIRED

- Micropipettes and a multichannel micropipette or multistep pipette, pipette tips
- Glass or plastic test tubes for diluting the samples.
- Laboratory bottles or beakers and graduated cylinders for diluting wash and dilution buffer
- Distilled or deionised water
- Absorbent paper towels
- Microtitre plate washer (alternatively, washing can be performed manually using a multichannel pipette)
- Microtitre reader equipped with a 450 nm filter and, if possible, a 620 nm reference filter
- Incubator (37 °C)

## 5. SAMPLES

Use fresh Citrate or EDTA plasma samples supplemented with platelet-stabilizing agents. Acidified citrate plasma samples can be used (final pH = 6). Collect the blood of patients to be tested in precooled plastic or siliconized tubes containing either sodium citrate, acidified sodium citrate or EDTA as anticoagulant as well as prostaglandin E<sub>1</sub> and theophyllin as platelet stabilizers in 1:10 ratio to blood. Commercially available tubes can be used. After filling, samples should be gently mixed by inverting the tube 5 times and then are placed in a crushed ice-water mixture. Centrifuge the blood within 90 minutes after the puncture at 2000 g for 30 minutes at 4 °C.

Pipette the pooled plasma into aliquots and store at a temperature below  $-30\text{ }^{\circ}\text{C}$ . Thawing and refreezing of plasma aliquots is not recommended.

Haemolytic and lipaemic plasmas may be used. Platelets contain PAI-1 and care should be taken to ensure that samples are platelet free. Serum samples should not be used since they generally show elevated PAI-1 values as compared to plasma. However, platelet PAI-1 is only to 5 % active and therefore does not contribute too much to active PAI-1.

## 6. TEST PERFORMANCE

### 6.1 PREPARATIONS AND STABILITY OF REAGENTS

All reagents must be at ambient temperature before use.

Component	Volume/ bottle	Additions	Bench Stability
Dilution buffer	20 ml	30 ml distilled $\text{H}_2\text{O}$	4-8 weeks at $4\text{ }^{\circ}\text{C}$
Wash buffer	20 ml	230 ml distilled $\text{H}_2\text{O}$	4-8 weeks at $4\text{ }^{\circ}\text{C}$
POX AB (concentrate)	-	10 ml sample dilution buffer	Aliquot $-20\text{ }^{\circ}\text{C}$ : 8 weeks RT: 4 hours
Standard 1 to 5 and Control Plasma A and B	-	0.2 ml distilled $\text{H}_2\text{O}$	Aliquot $-70\text{ }^{\circ}\text{C}$ : 8 weeks RT: 4 hours

RT = Room Temperature

POX AB = Peroxidase conjugated Antibody

#### MICROTITRE PLATE:

Unused strips reseal in aluminium foil bag. Store at  $4\text{ }^{\circ}\text{C}$  for up to 8 weeks.

### 6.2 SAMPLE DILUTIONS

Standards, controls and plasma samples are added directly to the plate but for abnormally high PAI-1 levels dilute 1:2

**50  $\mu\text{l}$  plasma + 50  $\mu\text{l}$  sample dilution buffer**

or dilute 1:4

**25  $\mu\text{l}$  plasma + 75  $\mu\text{l}$  sample dilution buffer**

### 6.3 ASSAY PROCEDURE

Overview of assay procedure

Time table Summary of procedure		<i>time required</i>	<i>Temp.</i>
<b>1. Wash plate 3 times</b>	250 $\mu\text{l}$		
<b>2. Addition of Sample and POX Antibody</b>	25 $\mu\text{l}$ 75 $\mu\text{l}$	<b>45 minutes</b>	$37^{\circ}\text{C}$
<b>3. wash 3 times</b>	250 $\mu\text{l}$		
<b>4. Substrate - incubation</b>	100 $\mu\text{l}$	<b>15 minutes</b>	RT
<b>5. Stop solution</b>	100 $\mu\text{l}$		
<b>6. Read absorbances at 450 nm/620nm</b>			
<b>Total time:</b>		<b>65 minutes</b>	

- **MICROTITRE PLATE RECONSTITUTION**

Wash required strips by adding 250  $\mu\text{l}$  of wash buffer to the wells and tip out the contents. Wash the strips twice further with wash buffer. Tap strips on absorbant paper and make sure the wells are completely dry.

- **SAMPLE/ STANDARD ADDITION**

Pipette 25  $\mu\text{l}$  of the samples/standard controls into separate wells. Running standard/control/sample in duplicate is recommended.

- **POX ANTIBODY ADDITION**

Pipette 75  $\mu\text{l}$  of POX antibody to all wells containing sample or standards/controls. Mix contents of well by tapping gently the side of the plate.

- **INCUBATION**

Cover the plate with a fresh plastic foil and incubate for **45 minutes** at  $37\text{ }^{\circ}\text{C}$ .

- **WASH PLATE**

Wash three times as described in step 1.

- **SUBSTRATE**

Pipette 0.1 ml of TMB substrate to all wells. Incubate for **15 minutes** at room temperature. There should be a colour change from clear to blue.

- **STOP**

Pipette 0.1 ml of stop solution to all wells. The colour of the endproduct should be yellow.

- **READ**

Measure absorbances at 450 nm (with 620 nm reference filter if available). Read absorbances within one hour after the addition of the stop solution.

### 6.4 NOTES FOR THE USER

Be sure to prepare all reagents before proceeding with the assay. It is critical to keep the time necessary for pipetting standards and samples to a minimum and avoid delays.

Be sure to wash the plate thoroughly and completely remove any residual wash buffer after each wash cycle. Insufficient washing can lead to erroneously high values and incomplete removal of wash buffer to irregularities due to the dilution of added reagents.

As mentioned use a multistepper to add peroxidase conjugate, TMB substrate and stop solution.

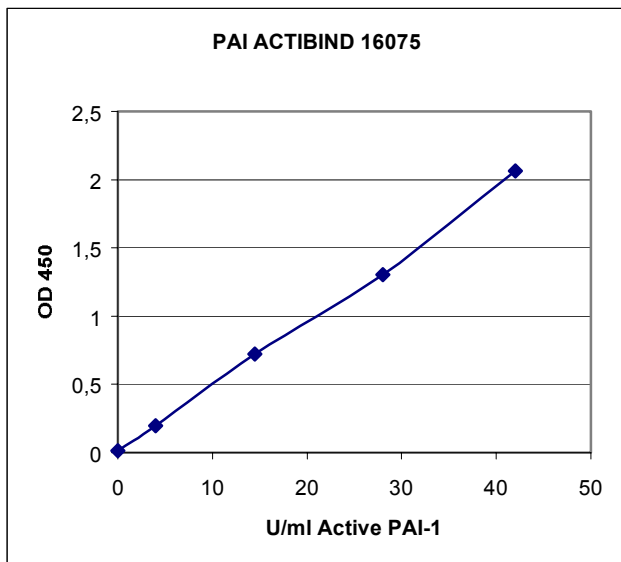
## 7. TEST EVALUATION

### 7.1 CONSTRUCT A STANDARD CURVE

Construct a graph of the standard curve. An example of a typical curve is given below. A standard curve must be constructed with each assay.

### 7.2 DETERMINATION OF SAMPLE CONCENTRATION

Locate the absorbance for each sample on the curve and read the corresponding value from the horizontal axis. For samples added directly to the plate no dilution factor multiplication is necessary. Do not forget to multiply by the dilution factor (2) or (4) for the samples, prediluted before adding to the plate.



## 8. EVALUATION OF RESULTS

Active PAI-1 levels above 20 U/ml may indicate reduced fibrinolytic capacity and, thus, increased thrombotic tendency. Measures should be taken to reduce the risk of thrombosis in individuals with elevated PAI-1 plasma levels.

Normal plasma levels range from 1-7 U/ml.

## 9. TEST CHARACTERISTICS

The TC Actibind-PAI-1 ELISA exclusively measures free, active PAI-1 and is not affected by other forms of PAI-1 or other plasminogen activator inhibitors. The assay range is 1 – 70 U/ml plasma. The inter- and intra-assay variations are less than 10 % and 5 %, respectively.

## 10. STABILITY AND STORAGE

All the components of the kit should be stored at 2...8°C and can be used until the indicated expiry date on the vial labels. For storage of samples see point 5.

## 11. SPECIAL PRECAUTIONS

Potentially biohazardous material. Donor plasma used in this kit was tested by internationally approved methods for the presence of antibodies to HIV and hepatitis B virus and found to be negative. However, all human blood products should be handled as potentially infectious material.

The stop solution ( $H_2SO_4$ ) can cause skin irritations, wash with plenty of water if spilled on the skin.

## References

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