ANIARA

Manufactured By: HYPHEN BioMed

ZYMUTEST PAI-1 Antigen Technical File

(# ARK012A)

Complete ELISA kit for tissue- Plasminogen Activator Inhibitor Type 1 (PAI-1).

Assay range: 0 to 10ng/ml

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May 2006

Form AH44
4-2007

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Assay principle

- The ZYMUTEST PAI-1 Antigen kit is a one step, two site immuno-assay for measuring human tissue-Plasminogen Activator Inhibitor, type 1 (PAI-1) in plasma, or in any fluid where PAI-1 can be present.

- First, the immunoconjugate, which is a monoclonal antibody specific for PAI-1 coupled to Horse Radish Peroxidase (HRP), is introduced into the microwells coated with another monoclonal antibody specific for PAI-1.

- Then, the diluted tested sample is immediately introduced, and the immunological reaction starts. When present, PAI-1 binds onto the monoclonal antibody coated solid phase through one epitope, and fixes the second monoclonal antibody coupled to HRP by another epitope.

- Following a washing step, the peroxidase substrate, 3,3',5,5' – TetraMethylBenzidine (TMB), in presence of Hydrogen Peroxide (H_2O_2), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained.

- The amount of colour developed is directly proportional to the concentration of human PAI-1: Ag in the tested sample.

Intended use: IVD   CE

- Assay of PAI-1: Ag in clinical samples.
- Measurement of PAI-1 as a cardiovascular risk factor.

Kit presentation: 96 tests (microplate)

- 1 micro ELISA plate (12x8wells)
- 2 vials of sample diluent
- 3 vials of calibrator (lyophilized)
- 1 vial of Control I (high, human plasma) (lyophilized)
- 1 vial of Control II (low, human plasma) (lyophilized)
- 3 vials of Immunoconjugate (lyophilized)
- 1 vial of conjugate diluent (ready to use)
- 1 vial of 20 fold concentrated wash solution
- 1 vial of substrate (ready to use)
- 1 vial of stop solution (ready to use)

Procedure

- Specimen: citrate or Na2EDTA anticoagulated human plasma.
- Plasma Dilution: 1:5 (or higher in presence of elevated PAI-1).
- Calibration: No international standard available at this time. Calibrator included, which concentration is defined (in ng/ml) against an internal standard (highly purified PAI-1 preparation, with an accurate PAI-1 concentration determined by Lowry, Bradford, A280 and A210 methods, and amino acid sequencing).
- Manual method or specific automates for ELISA.

## Calibration curve

PAI-1 Concentration (ng/ml)
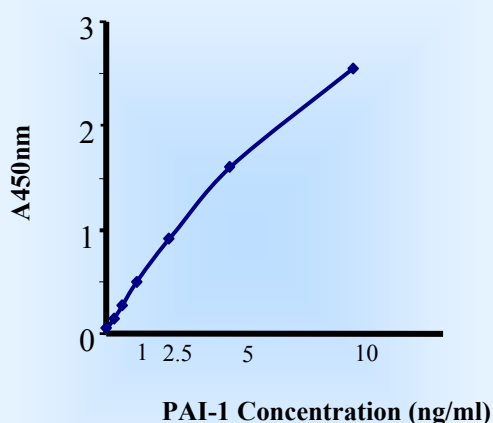
The assay has a dynamic range from 0 to 10 ng/ml in the tested dilution (calibration curve from 0 to 10 ng/ml, i.e. 0 to 50 ng/ml in plasma assayed diluted 1:5).

Assay Characteristics

- Total assay time : **about 1h 05min** (alternative **two-step procedure** in about 2h 15min)

- Assay range : **0 to 10 ng/ml** PAI-1 Antigen in the tested dilution (ie **0 to 50ng/ml in plasma tested diluted 1:5**).

- Detection threshold (blank+3SD, N≥10): **≤ 0.5 ng/ml**

- Reproducibility: Intra assay CV **3 to 8 %**
  Inter assay CV **5 to 10%**

- No significant interference of heparin up to 2 IU/ml.

- **Specificity:** This monoclonal antibody based assay, has homogeneous reactivity to the various forms of PAI-1, latent, active, bound to vitronectin, complexed to tPA, or to uPA, or inactive.

- Insensitive to presence of Rheumatoid factor.

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Stability of reconstituted reagents

Stability of reconstituted immunoconjugate

Legend: 24h/RT, Fresh(1), 4weeks/2-8°C, Fresh(2)
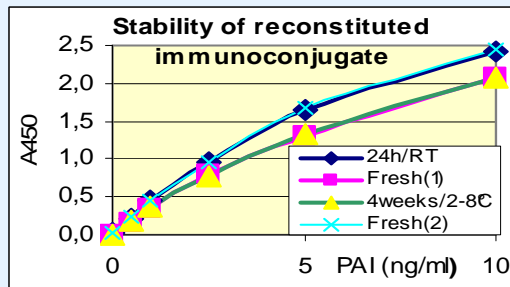
Standard 10ng/ml A450 :	
Fresh	2.08
12h/RT	2.09

Controls (ng/ml)	Fresh	24h 2-8°C	12h RT
CI	38.0	37.6	35.3
CII	11.0	11.3	10.8

Excellent preservation of performances for reconstituted reagents stored at 2-8°C or at RT (according to the device insert), comparatively to freshly reconstituted vials.

Overheating study

Calibration curve

Legend: 30°C, 2-8°C
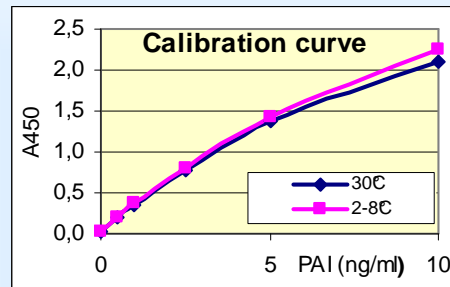
Controls (ng/ml)	2-8°C	30°C
CI [33-43]	33.5	35.9
CII [10.7-14.7]	11.5	11.2

Excellent preservation of performances in "overheating studies" of lyophilised products stored for 3 weeks at 30°C comparatively to those kept at 2-8°C.
Kits can be shipped at RT for a short period without any damage.

Real time follow up of reagents at 2 – 8 °C

Calibration curve

Legend: Initial QC, Expiration
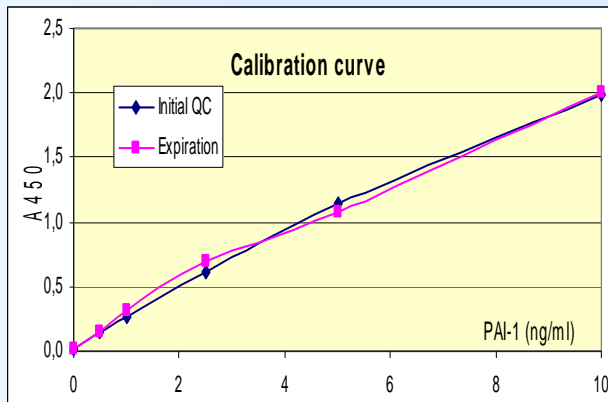
Measured PAI-1:Ag concentrations in controls (ng/ml)		
	Initial QC release	Expiration date (after 30 months at 2-8°C)
CI [26.0-31.7]	28.8	28.5
CII [7.7-10.4]	9.0	9.2

Excellent performances preservation of reconstituted reagents following storage of lyophilized products for 30 months at 2-8°C (control at the expiration date).

Intra- and inter-assay reproducibilities

Calibration curve reproducibility:

STANDARD (ng/ml) (N= 6 vials)	Mean A450	CV%
10	1.99	2.7
5	1.15	4.1
2.5	0.61	4.4
1	0.27	3.3
0.5	0.15	4.1
0	0.02	/

Conclusion:
Expected reproducibility values are obtained
(intra assay CV 3 to 8%, inter assay CV 5 to 10%).

Intra-assay reproducibility: Tested by measuring samples 10 times in the same series (N=10):

Sample	PAI-1 Mean Concentration (ng/ml)	SD	CV (%)
CI	36.7	1.29	**3.5**
CII	8.5	0.26	**3.0**

Inter-assay reproducibility: Tested by aliquoting samples, deep freezing them at -80°C, and testing them in 10 separate series.

Sample	PAI-1 Mean Concentration (ng/ml)	SD	CV (%)
CI	35.6	3.25	**9.1**
CII	8.4	0.82	**9.7**

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Specificity: Recovery study

Native recombinant-human-PAI-1 is spiked into normal citrated plasma and in plasma with high PAI-1 concentrations, diluted in the diluent without or with tPA (for testing reactivity to PAI-1complexed with tPA) and then tested with Zymutest PAI-1:Ag.

Plasmas	Sample diluent				Sample diluent + tPA			
	Measured plasma PAI-1 concentration (ng/ml)		Expected PAI-1 (ng/ml)	Recovery (%)	Measured plasma PAI-1 concentration (ng/ml)		Expected PAI-1 (ng/ml)	Recovery (%)
	initial plasma	with addition of PAI-1 50ng/ml final			initial plasma	with addition of PAI-1 50ng/ml final		
Mean (N=10)	16.6	64.4	66.6	97	19.4	67.7	69.4	98

Conclusions: native PAI-1 is accurately recovered following its addition to plasma, whether tPA is absent or present in the sample diluent. Free PAI-1 and PAI-1 complexed to tPA are therefore measured similarly with the ZYMUTEST PAI-1 Ag kit. Besides, PAI-1 is bound to vitronectin in plasma, and the recovery obtained confirms that this PAI-1 form is correctly measured.

Heparin interference study

Heparin is spiked at various concentrations (from 0 to 2 IU/ml final) into 3 different normal plasmas. The PAI-1 Ag concentration is then measured:

Plasmas	Heparin concentration added to plasma:				
	0 IU/ml	0.5 IU/ml	1 IU/ml	1.5 IU/ml	2 IU/ml
	(ng/ml PAI-1)				
1	31.6	33.7	35.9	37.6	37.7
2	8.0	8.0	8.6	8.7	9.3
3	19.9	19.1	18.9	19.4	19.1

Conclusion: There is no significant effect of heparin up to 2 IU/ml on the PAI-1 Ag concentrations measurement in plasmas, when using the Zymutest PAI-1 Antigen device.

Comparison of Performances: One step / Two steps method for ZYMUTEST PAI1:Ag

	One step method	Two steps method
Mean PAI-1 Ag (ng/ml) : (N=32 plasmas)	6.62	6.32

One step vs Two steps method
(N=32 , y = 0.91x + 0.33)
$R^2 = 0,9846$
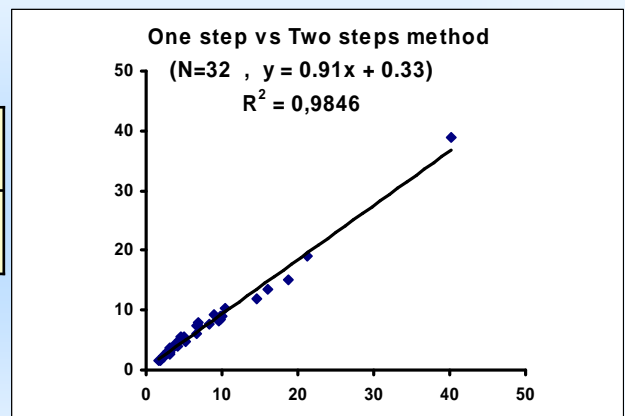
Conclusion: Excellent correlation between the 2 methods.

Comparison of PAI1:Ag measurement s in buffer or plasma milieu

61 normal plasma samples were tested with the ZYMUTEST PAI-1:Ag device, using a calibration curve realised with PAI-1 spiked into a "buffer milieu" or in "plasma":

Calibration in:	«buffer»	« plasma»
Measured PAI-1:Ag values for Normal Plasmas (in ng/ml)		
N (samples)	61	61
Mean	12.6	14.2
Median	10.1	11.4
SD	8.4	9.8
Minimum	2.6	2.8
Maximum	39.1	45.7

Linear regression analysis: PAI-1 Ag measurement in purified buffered milieu compared with in plasma milieu
(N=61 ; R =0.999 ; R2 = 0.983 ; Y = 1.13X)


$y = 1,1343x - 1E-14$
$R^2 = 1$
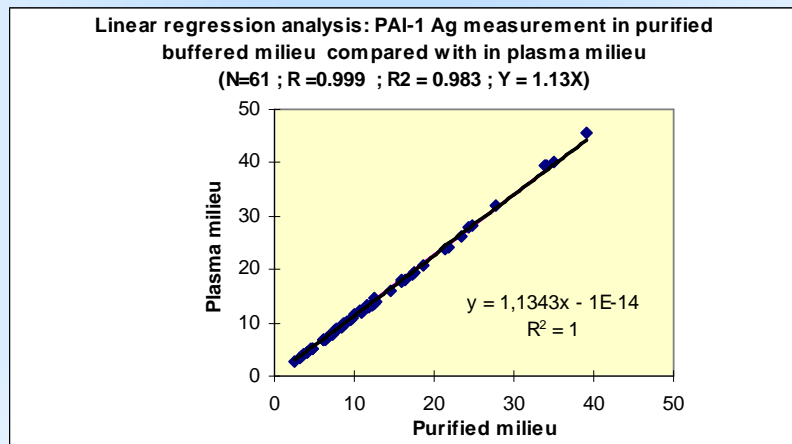
Conclusion:
Correlation results are excellent; and there is only a slight difference between PAI-1:Ag concentration measured in buffer milieu or plasma milieu (difference ≈10%). Calibration is made in plasma for the Zymutest PAI-1 Ag kit.

Correlation study between PAI-1:Ag, PAI-1 Activity and tPA:Ag
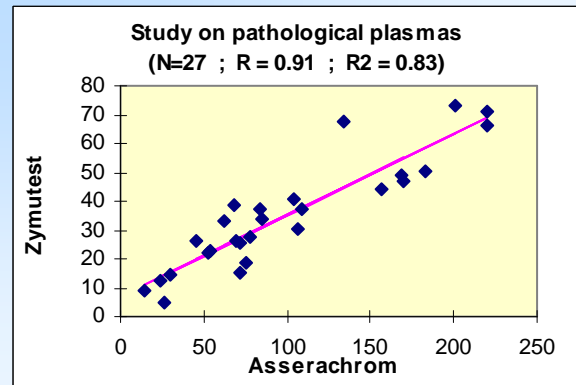## (253 normal individuals and patients with carotid stenosis)

	Zymutest PAI-1:Ag	Zymutest PAI-1 Act.	Zymutest tPA:Ag
N	253	253	253
Mean	14.23 ng/ml	1.08 ng/ml	6.42 ng/ml
SD	12.31 ng/ml	1.28 ng/ml	2.63 ng/ml

Y = 8,39x + 5,20
R2 = 0,76 ; R = 0,87

Y = 2,34x - 0,798
R2 = 0,25 ; R = 0,50
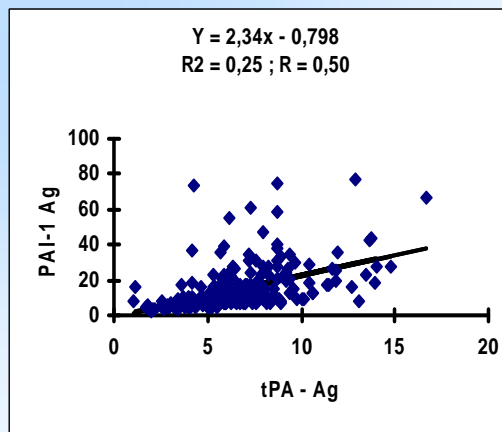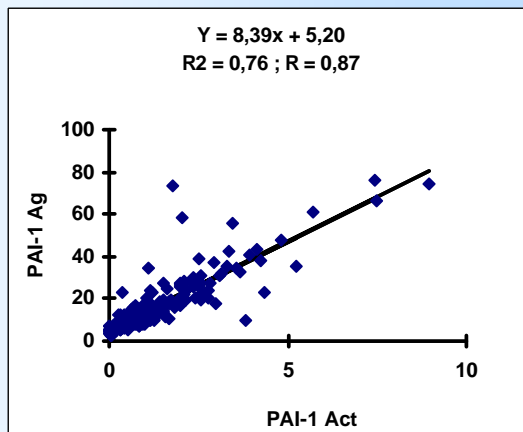
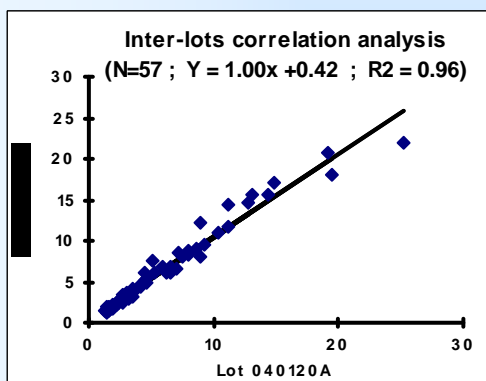# ZYMUTEST PAI-1:Ag technical file (Ref ARK012A)

Comparison of Performances with a commercial device (Asserachrom PAI-1)

	Zymutest PAI-1 Ag Lot 040120A (ng/ml)	Asserachrom PAI-1 Ag Lot 031731 (ng/ml)
Normal Plasmas		
N	46	46
Mean	8.9	15.0
SD	7.8	15.5
Pathological Plasmas (oncology, dicoumarol therapy, pathological pregnancy)		
N	27	27
Mean	34.5	102.6
SD	18.7	62.4

Study on normal plasmas
(N=46 ; Y= 0.47x+1.76 ; R=0.94 ; R2=0.88)

Study on pathological plasmas
(N=27 ; R = 0.91 ; R2 = 0.83)
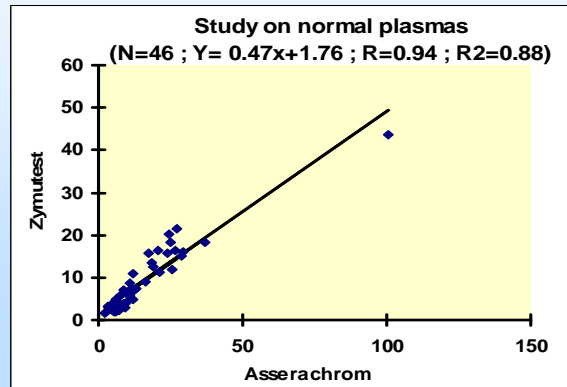
Conclusions: Mean values (absolute value) are different, as reference material is different for both kits, but correlations are excellent between the 2 devices.
Nota: A multicentric study (standardisation subcommittees, fibrinolysis congress, published in *Thrombosis and Hemostasis* (1993 Nov ; 70(5) : 858-63) was organized to evaluate plasma PAI-1:Ag measurement results obtained from different kits provided by different suppliers. A very important variation was noticed between devices (Mean PAI-1 conc.). According to the assay used, average values in normal plasma ranged from 7.4 to 28 ng/ml). Correlation coefficients obtained with the different kits were between 0.973 and 0.999. The definition of the reference PAI-1 material and concentration used for calibrating the assays explains the differences observed. Furthermore, the various assays might measure differently the different PAI-1 presentations.

Inter-lots performances comparison of ZYMUTEST PAI-1:Ag device, on normal and pathological samples.

Inter-lots correlation analysis
(N=57 ; Y = 1.00x +0.42 ; R2 = 0.96)

ZYMUTEST PAI-1 :Ag	Lot 030311E	Lot 040120A
	PAI-1 (ng/ml)	PAI-1 (ng/ml)
Normal plasmas		
N	57	57
Mean (ng/ml)	6.6	6.1
SD	5.2	5.1
Minimum - Maximum	1.2 - 21.8	1.3 - 25.3
Pathological plasmas (pathological pregnancy, oncology, intensive care, dicoumarol therapy)		
P1	24.3	21.3
P2	9.5	7.2
P3	25.0	25.1
P4	14.1	10.9
P5	18.0	10.4
P6	90	74
P7	10.9	9.9
P8	27.8	28.2
P9	> 50	> 50
P10	> 50	> 50
P11	> 50	> 50
Controls		
CI [26.00-31.70]	28.2	30.0
CII [7.70-10.40]	9.1	9.6
CI [34.60-42.40]	39.0	42.0
CII [11.30-15.30]	15.0	15.0

Conclusion:
Excellent correlation between the 2 lots. Performances of ZYMUTEST PAI-1:Ag device are homogeneous between the various manufacturing lots.

Clinical applications

• PAI-1 is a single chain glycoprotein, synthesized by endothelial cells and hepatocytes and with a molecular weight of 50,000 daltons. In plasma it is stabilised by binding to vitronectin, or circulates as inactive complexes with tPA or uPA. PAI-1 is also present in platelets, but in the latent form. PAI-1 regulates fibrinolysis by inhibiting tPA or urokinase.

• The normal range for PAI-1 : Ag concentrations tested with Zymutest PAI-1 Ag is from 1 to 25 ng/ml.

(Nota: in the absence of reference material for PAI-1:Ag assays, there is an important heterogeneity of PAI-1 concentrations measured with the various commercial assays. The normal range must be considered respectively to the device used (6)).

• PAI-1 increases with age and lipid metabolism, especially with triglyceride concentration. PAI-1 presents diurnal variations, the highest concentrations being measured at morning. PAI-1: Ag concentration increases during pregnancy.

• PAI-1 Antigen is increased in thrombosis, malignant diseases, hepatic disorders, post surgical period, sepsis. It is also elevated in malignant tissue extracts. Recent studies have demonstrated a relationship between increased PAI-1 concentrations and cardiovascular risk factors (obesity, hyperinsulinemia, hypertriglyceridinemia, atherothrombosis...).

Application ⟹ **Assay of PAI-1: Ag in clinical samples.**
Measurement of PAI-1 as a cardiovascular risk factor.

References:

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2) Loskutoff D.J., Samad F. : The adipocyte and hemostatic balance in obesity. Studies on PAI-1. Artheriioscl. Thromb. Vasc. Biol., 1998, 18, 1-6.
3) Fujii S. : PAI-1 in Thrombosis and Arteriosclerosis. Fibrinolysis and Proteolysis, 1997, 11, 137-140.
4) De Maat MPM, De Bart A.C.W., Hennis BC, Meijer P., Havelmaar AC, Mulder PG, Kluft C. : Interindividual and Intraindividual variability in plasma Fibrinogen, tPA antigen, PAi-1 Activity and CRP in healthy, young volunteers and patients with angina pectoris. Arterioscl. Thromb. Vasc. Biol., 1996, 16, 1156-62
5) Smith F.B., Lee A.J., Rumley A., Fowkes G.R., Lowe G.O.R. : Tissue-Plasminogen-Activator, Plasminogen Activator Inhibitor and risk of peripheral arterial disease. Artheriosclerosis, 1995, 115, 35-43.
6) Declerck PJ et al., Multicenter evaluation of commercially available methods for the immunological determination of plasminogen activator inhibitor-1 (PAI-1), Thromb. Haemost., 1993, 70(5), 858-63.