ANIARA DIAGNOSTICA LLC

Please note that the uses described in the following page(s) have not been approved or cleared by FDA, with respect to the described assay or test.

In the US, the product is intended **For Research Use Only. Not for Use in Diagnostic Procedures.**

Aniara Diagnostica LLC • 7768 Service Center Drive • West Chester, OH 45069 • Phone (513) 770-1991
Fax (513) 573-9241 • Email info@aniara com • www.aniara.com

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Manufactured By: HYPHEN BioMed

ZYMUTEST tPA Antigen Technical File

(# ARK011A)

Complete ELISA kit for Tissue-Type Plasminogen Activator Antigen.

Assay range on plasma: 0 to 20ng/ml

CE

May 2006

Form AH43
4-2007

ANIARA 6560 Gove Court · Mason, OH 45040
Phone: 513.770.1991 Toll Free: 866.783.3797 Fax: 513.573.9241 Email: info@aniara.com
www.aniara.com

ZYMUTEST tPA:Ag technical file (Ref ARK011A)

Assay principle

- The ZYMUTEST tPA kit is a two-site immuno-assay for measuring human tissue- Plasminogen Activator (tPA) in plasma, or in any fluid where tPA can be present.
- In a first step, the diluted tested plasma or biological fluid is introduced into a microwell coated with a highly purified monoclonal antibody specific for human tPA.

When present, this protein is captured onto the solid phase.

Following a washing step, the immunoconjugate, which is a monoclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and binds to another free epitope of immobilized tPA.

Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H2O2), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with sulfuric acid.

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

Intended use: IVD

CE

- Assay of tPA:Ag in clinical samples, as a disease marker, or for epidemiological studies.
- Assay of tPA:Ag in thrombolysis with recombinant tPA drugs.

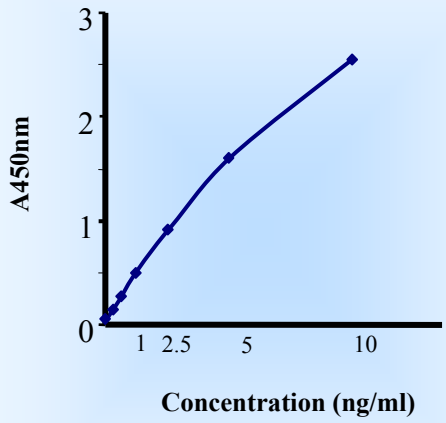
Kit presentation: 96 tests (microplate)

- 1 microELISA plate (12x8wells)
- 2 vials of sample diluent
- 3 vials of calibrator (lyophilised) (concentration defined according to the NIBSC International Standard for tPA).
- 1 vial of Control I (high, human plasma) (lyophilised)
- 1 vial of Control II (low, human plasma) (lyophilised)
- 3 vials of immunoconjugate (lyophilised)
- 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: 1:2.
- Calibration: calibrator included, which concentration is defined (in ng/ml) using the NIBSC international standard preparation for tPA.
- Manual method or specific automates for ELISA.

Calibration curve



The assay has a dynamic range from 0 to 10ng/ml, in the tested dilution, or from 0 to 20 ng/ml on plasma, assayed twofold diluted.

Assay Characteristics

- Total assay time : **about 2h 15min (for the « two steps » asssay)** (« **one step** » **rapid procedure** in **about 1h 15min**)
- Assay range : **0 to 20 ng/ml** tPA Antigen in plasma
- 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
  - endogeneous PAI-1 up to 100 ng/ml
- **Specificity:** The kit, designed with 2 specific monoclonal antibodies, allows measuring homogeneously tPA in plasma, whether its presentation is, free and active or complexed with its inhibitors (tPA-PAI-1 complexes).
  A good recovery is also obtained when purified tPA is added to normal plasma.
- Insensitive to the Rheumatoid factor.

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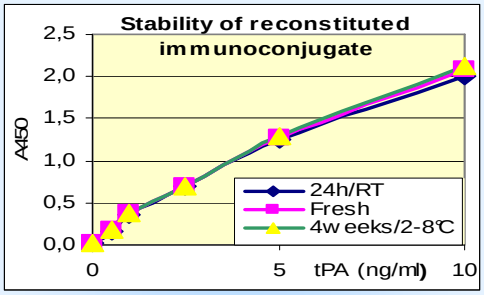
Stability of reconstituted reagents

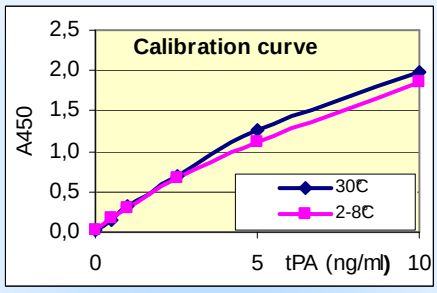


Standard 10ng/ml A450 :	
Fresh	2.71
12h/RT	2.68

Controls (ng/ml)	Fresh	24h 2-8°C	12h RT
CI	7.12	6.92	7.13
CII	2.51	2.60	2.51

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

Overheating studies

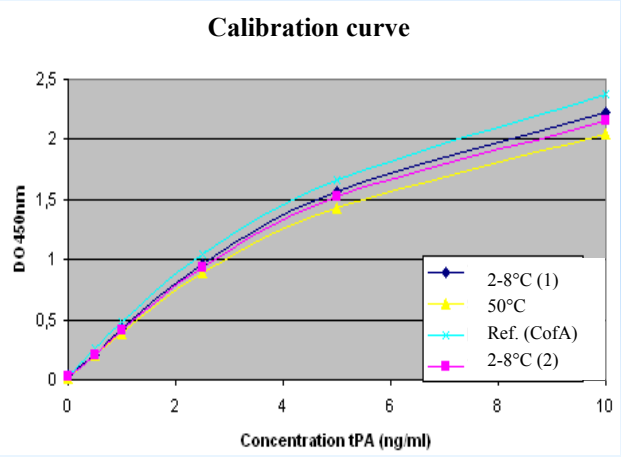


Controls (ng/ml)	2-8°C	30°C
CI [9.0 - 11.4]	11.0	10.1
CII [2.5 - 3.5]	2.8	3.3

Excellent preservation of performances following storage of lyophilised reagents 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 damage.

Real-time follow-up at 2-8°C, and Heat stressing study at 50°C



Measured tPA :Ag concentrations for the controls (in ng/ml)				
	2-8°C (1)	50°C	*Ref (CofA)*	2-8°C (2)
CI [8.7-10.7]	8,87	8,97	*9,5*	8,85
CII [2.3-3.3]	2,3	2,43	*2,9*	2,77

Excellent preservation of performances following storage of lyophilised products for 6 months at 2-8 °C, and then 3 days at 50°C or 2-8°C.
It further confirms that these kits can be shipped at RT for a short period without damage.

Intra- and inter-assay reproducibilities

Calibration curve reproducibility:

STANDARD (ng/ml) (N=6 vials)	Mean A450	CV%
10	2.71	1.50
5	1.66	1.64
2.5	0.93	1.18
1	0.40	2.80
0.5	0.21	3.32
0	0.01	/

Conclusion: Expected reproducibility values are obtained (intra assay CV 3 to 8%, inter assay CV5 to 10%).

Intra-assay reproducibility: Tested by measuring samples 12 times in the same series (N=12):

Sample	tPA Mean Concentration (ng/ml)	SD	CV (%)
CI	12.48	0.65	**5.1**
CII	3.04	0.11	**3.6**

Inter assay reproducibility: Tested by aliquoting samples, deep freezing them at -80°C, and testing them in duplicate in 12 separate series.

Sample	tPA Mean Concentration (ng/ml)	SD	CV (%)
CI	11.85	0.95	**8.0**
CII	3.00	0.11	**3.7**

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ZYMUTEST tPA:Ag technical file (Ref ARK011A)

Specificity: Recovery study

r-tPA (actilyse) is spiked into normal citrated plasma or into plasmas with a high PAI-1 concentration, then tested with the Zymutest- tPA Ag kit (at the 1:2 dil.).

	Concentration of tPA added to plasma			
	0 ng/ml	10 ng/ml	25 ng/ml	60 ng/ml
Normal Plasma1	1.54	11.48	25.60	56.00
Normal Plasma 2	5.00	14.24	27.00	56.00
Pathological Plasma 1 Concentration of PAI-1 >100 ng/ml	50.00	61.00	77.00	102.00
Pathological Plasma 2 Concentration of PAI-1 = 87 ng/ml	3.98	15.00	32.00	58.00

Conclusion: This study shows a good recovery of r-tPA spiked in plasmas. In plasmas with a high PAI-1 concentration, the recovery is also good; Zymutest tPA kit recognizes free tPA as well as tPA complexed with PAI-1.

Heparin interference study

Heparin (from 0 to 2 IU/ml final concentration) is spiked into various normal or pathological plasmas.
The tPA concentration is then measured:

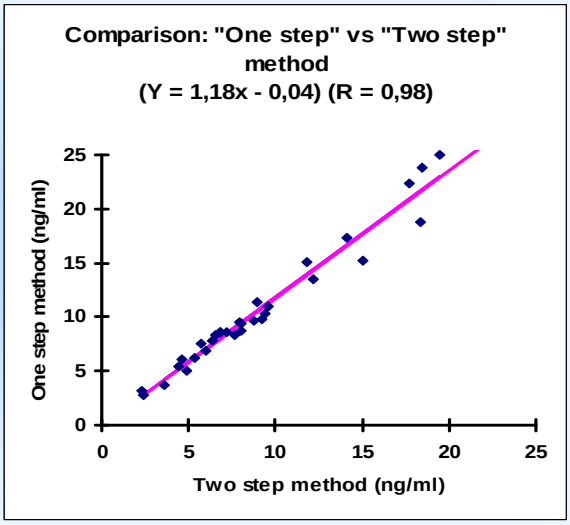
Conclusion:
There is no significant effect of heparin up to 2 IU/ml on the tPA concentrations measured on normal plasmas and pathological plasmas.

	Concentration of heparin added		
	0 IU/ml	1 IU/ml	2IU/ml
Normal Plasmas (ng/ml tPA)			
1	3.38	3.26	3.06
2	9.72	9.33	9.27
3	6.5	5.34	5.35
Pathological Plasmas (ng/ml tPA)			
1	52.00	47.00	49.00
2	10.30	10.40	10.00
3	11.70	11.10	9.94

Performances comparison: One step / Two step method for ZYMUTEST tPA

	Two step method	One step method
Pathological samples (pathogenic pregnancy, intensive care, oncology, cirrhosis)		
N	25	25
Mean tPA (ng/ml)	9.10	10.91
SD	0.99	1.22
« Normal » samples (hospital origin, without selection)		
N	6	6
Mean tPA (ng/ml)	11.58	12.70
SD	2.70	3.24

Conclusion: Excellent correlation between the 2 methods.



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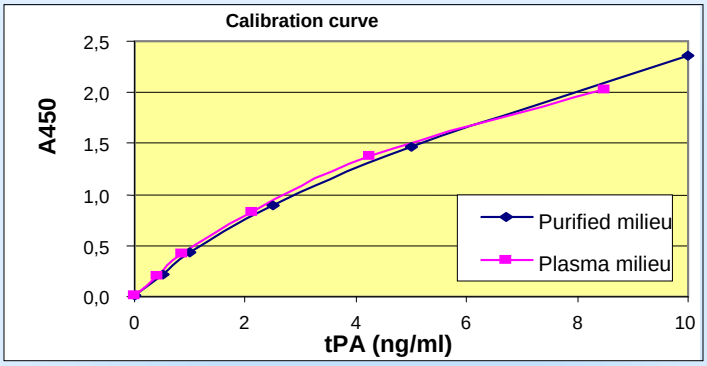
Performances comparison for tPA measurements in purified milieu or in plasma

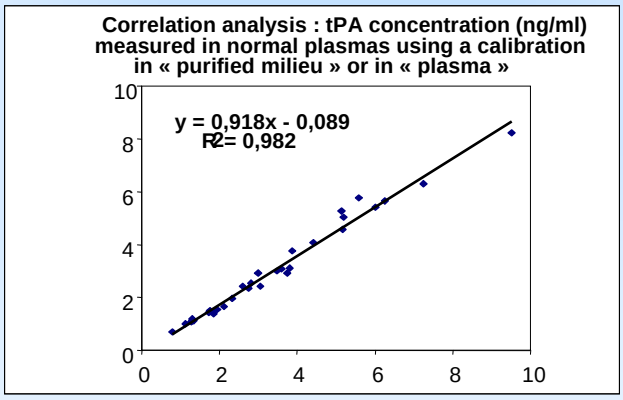
32 normal plasma samples were tested with the ZYMUTEST tPA:Ag device, using a calibration curve realised with tPA spiked into a "buffer milieu" or into a "plasma":

*** Calibration curves:**



*** Results obtained for normal samples:**

Calibration in:	«buffer milieu »	« plasma »
Measured tPA:Ag values for Normal Plasmas (in ng/ml)		
N (samples)	32	32
Mean	3.37	3.00
SD	2.05	1.90



Conclusion:

Correlation results are satisfying; and there is no significant difference between tPA concentration measured in a purified milieu or plasma milieu (difference <10%).

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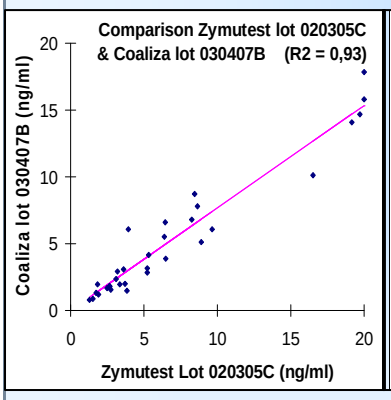
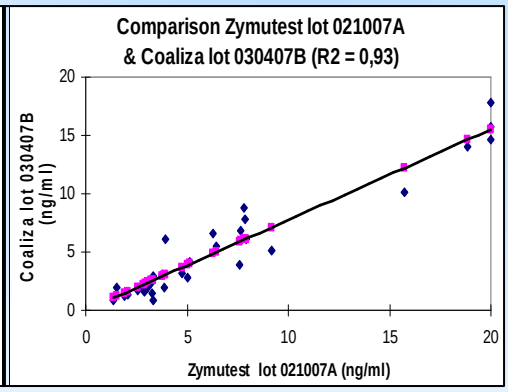
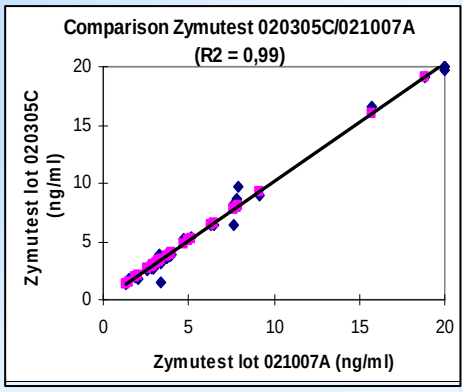
ZYMUTEST tPA:Ag technical file (Ref ARK011A)

Comparison with a commercial device (COALIZA) for tPA:Ag assay

	ZYMUTEST tPA Lot 020305C	ZYMUTEST tPA Lot 0201007A	COALIZA tPA Lot 030407B
Normal samples			
N	24	24	24
Mean measured tPA concentration (ng/ml)	**4.31**	**4.20**	**3.28**
SD	2.44	2.06	2.29
Pathological samples			
N	7	7	7
Mean measured tPA concentration (ng/ml)	**14.34**	**14.42**	**10.95**
SD	6.33	6.10	5.04



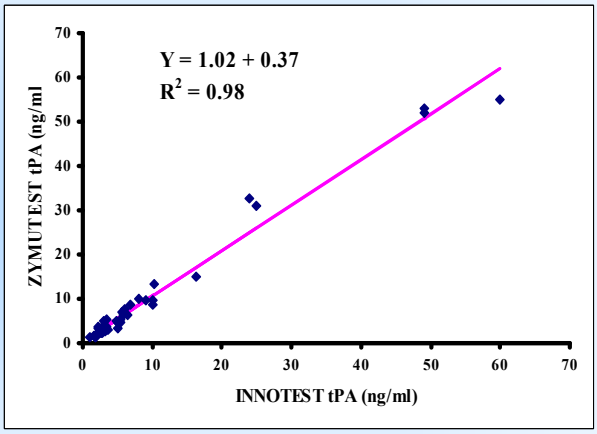




Conclusion: Good correlation between the 2 devices. Moreover, the good inter-lots performances reproducibility for ZYMUTEST tPA kits is confirmed.

Performances comparison with a commercial device (INNOTEST) for tPA assay

	Zymutest tPA (ng/ml)	Innotest tPA (ng/ml)
Normal Plasmas		
N	34	34
Mean	4.52	4.22
SD	2.75	2.29
Pathological Plasmas		
N	8	8
Mean	32.12	30.27
SD	18.38	18.41



Conclusion: Excellent correlation between the 2 devices.

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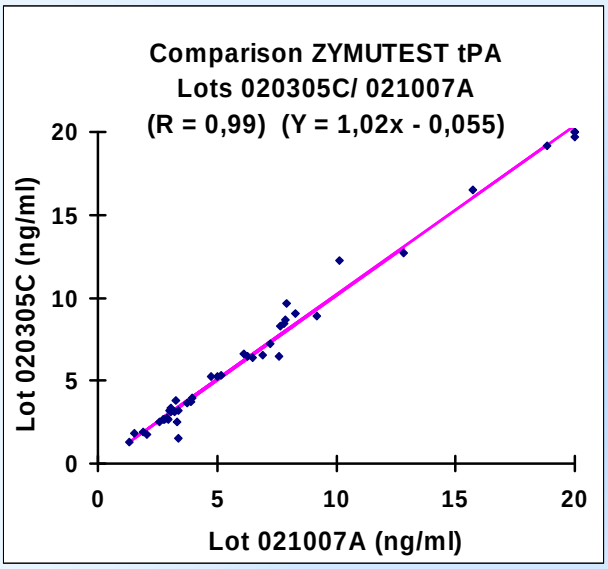
ZYMUTEST tPA:Ag technical file (Ref ARK011A)

Inter-lot performance comparison for the ZYMUTEST tPA device, on normal and pathological samples.



Specimen	Lot 020305C	Lot 0201007A
	tPA (ng/ml)	tPA (ng/ml)
Normal samples		
N	30	30
Mean	4.80	4.66
Standard Deviation	2.84	2.56
Pathological samples		
N	8	8
Mean	14.34	14.41
SD	6.33	6.10

Controls	Lot 020305C	Lot 0201007A
CI [10,04-13,58]	12,3	10,1
CII [2,41-3,61]	3,1	3,1
CI [6,77-8,28]	7,2	7,2
CII [2,22-3,01]	2,7	2,8

Conclusion:
Excellent correlation between the 2 lots.
Performances of ZYMUTEST tPA Antigen device are homogeneous between the various manufactured lots.

Clinical applications

•Tissue-Type Plasminogen Activator (tPA), is a 68 kDa protein, synthesised and secreted by endothelial cells.
It initiates fibrinolysis by activating plasminogen to plasmin onto the fibrin clot surface. It is composed of 563 amino acids.
In blood, tPA is rapidly inactivated by its major inhibitor PAI-1, which is usually in excess. Circulating tPA is then present predominantly in an inactive stable complex with PAI-1. Clearance of tPA is biphasic, phase 1 having a half-life of about 5 minutes and phase 2 a half-life of about 45 minutes. It binds to receptors on liver.
•The tPA:Ag concentration in normal human plasma is usually < 10 ng/ml. It increases with age, exercise and stress.
•Elevated concentrations of tPA are observed in various pathological conditions (respiratory distress syndrome, myocardial infarction, septicaemia, stroke, liver diseases, etc….). During liver transplantation, tPA concentrations are dramatically increased in the anhepatic phase.
Recent epidemiological studies have demonstrated an association between tPA:Ag concentrations and an increased risk of cardiovascular diseases. Elevated tPA:Ag is frequently associated with high PAI-1 concentrations and a decreased basic fibrinolytic potential. Assay of tPA:Ag is then of predictive value for cardiovascular pathology and disease evolution.

⇒ **Assay of tPA:Ag in clinical samples, as a disease marker.**
Assay of tPA:Ag in thrombolysis with recombinant tPA drugs.

References:

1)Bos R., Siegel K., Otter M., Nieuwenhuizen W: "Production and characterization of a set of monoclonal antibodies against Tissue-Type Plasminogen Activator (tPA)". *Fibrinolysis*, 1992; 6: 173-182.

2)Bos R., Hoegee-de Nobel E., Laterveer R., Meyer P. and Nieuwenhuizen W. "A one step enzyme immunoassay for the determination of total tissue-type plasminogen activator (tPA) antigen in plasma". *Blood Coag Fib*,1992; 3: 303-307.

3)Juhan-Vague I., Alessi M.C.: "Fibrinolysis and risk of coronary artery disease". *Fibrinolysis,* 1996; 10(3): 127-136.

4)Stein P., Heins M., Schoebel F.C., Pels K., Jax T.W., Stiegler H., Reinauer H., Strauer B.E., Leschke M. "Activation of the fibrinolytic system in patients with coronary artery disease and hyperfibrinogenemia". *Thromb Haemost*, 1997; 77(5): 970-974.

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