

ZYMUTEST tPA – PAI-1 complexes

RK017A

(Complete ELISA kit for measurement of tPA-PAI-1 complexes)

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTENDED USE:

The ZYMUTEST tPA-PAI-1 kit is a two-site immuno-assay for measuring complexes of human tissue plasminogen Activator (tPA) with its major inhibitor PAI-1, in plasma or in any fluid where tPA-PAI-1 complexes can be present.

This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

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, tPA – PAI-1 complexes are captured onto the solid phase through the tPA moiety. Following a washing step, the immunoconjugate, which is an anti-PAI-1 monoclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and binds to its specific epitope on PAI-1 present in tPA-PAI-1 complexes. 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-PAI-1 complexes in the tested sample.

TEST SAMPLE:

- Trisodium Citrate or Na₂ EDTA anticoagulated human plasma.
- Any biological fluid where tPA-PAI-1 complexes must be measured.

REAGENTS:

1. **COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with a highly purified murine monoclonal antibody specific for human tPA, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
 2. **SD:** 2 vials containing 50ml of F-Sample Diluent, ready to use.
 3. **Cal:** 3 vials of tPA-PAI-1 Calibrator, lyophilised. Each vial, when restored with 2 ml of F-Sample diluent (SD), allows obtaining the calibrator plasma. The exact tPA-PAI-1 concentration is indicated on the flyer provided in the kit.
 4. **CI:** 1 vial containing 0.5 ml of lyophilised Plasma Control I (High) (human plasma).
 5. **CL:** 1 vial containing 0.5 ml of lyophilised Plasma Control II (Low) (human plasma).
- Note:** The tPA concentrations and acceptancy ranges for controls can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.
6. **IC:** 3 vials of Anti-(h)-PAI-1-HRP immunoconjugate, a monoclonal antibody coupled to HRP, lyophilised.
 7. **CD:** 1 vial of 25 ml of Conjugate Diluent, ready to use.
 8. **WS:** 1 vial of 50 ml of 20 fold concentrated Wash Solution.
 9. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' – Tetramethylbenzidine containing hydrogen peroxide. Ready to use.
 10. **SA:** 1 vial of 6 ml of 0.45M Sulfuric acid (Stop solution). Ready to use.

Note: Use only components from kits with the same lot number. Do not mix components from different lots of kits when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- 8-channel or repeating pipette allowing dispensing 50-300 µl.
- 1-channel pipettes at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to 1000 µl.
- Micro ELISA plate washing equipment and shaker.
- Micro ELISA plate reader with a wavelength set up at 450 nm.
- Distilled water.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amount of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 4 weeks in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
2. **F-Sample Diluent:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
3. **tPA-PAI-1 Calibrator:** restore each vial with 2 ml of F-Sample Diluent in order to obtain a solution of tPA-PAI-1 complexes. This solution is stable for at least 8 hours at room temperature.
4. **Plasma Control I (human plasma, High):** restore with 0.5 ml distilled water.
5. **Plasma Control II (human plasma, Low):** restore with 0.5 ml distilled water.

Note: when restored, plasma controls I and II are stable for 8 hours at room temperature, 24 hours at 2-8°C or 2 months frozen at -20°C or below.

Warning: Plasma controls I and II (4&5) are prepared with normal human plasma. This latter was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

6. **Anti-(h)-PAI-1-HRP immunoconjugate:** each vial must be restored with 7.5 ml of Conjugate Diluent. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2-8°C.
7. **Conjugate Diluent:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
8. **Wash Solution:** Incubate the vial for 15-30 minutes in a water bath at 37°C until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow preparing 1 liter of Wash Solution). The Wash Solution must be stored at 2-8°C in its original vial and used within 4 weeks following opening. The diluted Wash Solution must be used within 7 days, when protected from any contamination and stored at 2-8°C. This reagent contains 0.05% Kathon CG.
9. **TMB substrate:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8°C, and provided that any bacterial contamination is avoided during use.
10. **Stop solution:** It is ready to use.

Cautions: Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

Note: Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

PROCEDURE:

Specimen collection:

Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.) by a clean venipuncture; plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within 4 hours or stored frozen at -20°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within 4 hours.

EDTA collected human plasma may also be used. Conditions of storage are the same than those for citrated plasma.

In order to avoid diurnal variations, tPA-PAI-1 complexes must be preferentially measured on fasting samples, collected in the morning.

Tested plasma or sample or controls:

The sample must be tested diluted **two fold (1:2)** in the F-Sample Diluent. For expected tPA-PAI-1 concentrations >20 ng/ml, plasma or samples can be tested at a higher dilution, **1:5, or 1:10, or more**.

Controls I and II must be tested diluted **two fold (1:2)**, with F-Sample Diluent.

Calibration:

Using the **tPA-PAI-1 complexes** with a concentration "C" indicated, for each lot of reagents, on the flyer provided in the kit, prepare the following standard solutions.

tPA-PAI-1 concentration (ng/ml)	C	C/2	C/4	C/10	C/20	0
Vol. of tPA-PAI-1 calibrator	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of F-Sample Diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenization. The standard dilutions are stable for at least **6 hours** at room temperature.

Assay procedure:

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
tPA-PAI-1 calibrator or tested sample or controls diluted 1:2 or F-sample diluent (blank)	200 µl	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well (a).
Incubate for 1 hour at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 5 successive washings using the washing instrument (c).
Conjugate (anti PAI-1 monoclonal antibody coupled with peroxidase. Restored with 7.5 ml of Conjugate Diluent)	200 µl	Introduce the Anti-(h)-PAI-1- HRP immunoconjugate in the micro ELISA plate wells (c).
Incubate for 1 hour at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 5 successive washings using the washing instrument (c).
TMB/H ₂ O ₂ Substrate	200 µl	Immediately after the washing, introduce the substrate into the wells. Note: The substrate distribution, row by row, must be accurate and at exact time intervals (c, d).
Incubate for exactly 5 minutes at room temperature (18-25 °C) (b)		
0.45M Sulfuric Acid	50 µl	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid.
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450) (e) . Subtract the blank values		

Note:

- a) The two fold dilutions can be performed directly into the reactive well by introducing 100 µl of F-sample diluent and 100 µl of tested plasma sample or control.
- b) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.
- c) Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
- d) For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
- e) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RAPID PROCEDURE (ONE STEP METHOD):

The assay can be performed with a one step method. In this case, the calibration curve is unchanged (from 0 to C), the tPA-PAI-1 calibrator being reconstituted with **2 ml** of F-Sample Diluent.

The immunoconjugate must be reconstituted with **2 ml** of Conjugate Diluent.

Tested plasma must be assayed at a **two fold (1:2)** dilution or at higher dilutions in F-Sample Diluent. In the microwell, 50µl of immunoconjugate (anti-(h)-PAI-1 peroxidase) are first introduced, then 200µl of the diluted tested specimen. Following a 1 hour incubation at room temperature and a washing step, the colour development with TMB is allowed to develop for 5 min, and is then stopped with 50 µl of sulfuric acid. The calibration curve is drawn as indicated in results. The tPA-PAI-1 concentrations read must be multiplied by the sample dilution factor.

RESULTS:

On a linear graph paper plot the **tPA-PAI-1 concentration** on abscissae and the corresponding absorbance on ordinates.

From the curve obtained, deduce the tPA-PAI-1 concentration for the tested sample. For obtaining the tPA-PAI-1 complexes concentration in this sample, the value measured must be multiplied by the dilution factor (i.e., **2,5,10,...**).

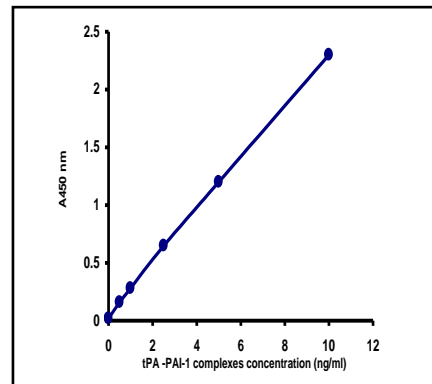
For **controls I and II**, the concentrations measured must be multiplied by **2**.

Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only. Users must construct their own calibration curve, obtained using their standard dilutions.



BIOCHEMISTRY:

Stoichiometric, stable tPA-PAI-1 complexes are generated when a tPA molecule binds to its major inhibitor PAI-1. The complexes have a MW of 120,000 daltons (70,000 for tPA and 50,000 for PAI-1), and are rapidly cleared from blood circulation. The concentration of tPA-PAI-1 complexes in normal human plasma is usually low (< 5 ng/ml).

REFERENCES:

Bos R. et al, "Production and characterization of a set of monoclonal antibodies against Tissue-Type Plasminogen Activator (tPA)". Fibrinolysis, 6: 173-182, 1992.