ZYMUTEST uPA – PAI-1 complexes

 # RK018A

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

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

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INTENDED USE:

The ZYMUTEST uPA-PAI-1 kit is a two-site immuno-assay for measuring complexes of human Urokinase Plasminogen Activator (uPA) with its major inhibitor PAI-1, in plasma or in any fluid where uPA-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 uPA. When present, uPA – PAI-1 complexes are captured onto the solid phase through the uPA 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 uPA-PAI-1 complexes. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H_2O_2), 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 uPA-PAI-1 complexes in the tested sample.

TEST SAMPLE:

- Trisodium Citrate or Na_2 EDTA anticoagulated human plasma.
- Any biological fluid where uPA-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 uPA, 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 **uPA-PAI-1 Calibrator**, lyophilised. When restored with 2 ml of F-Sample Diluent, a solution containing "C" (ng/ml) of human uPA-PAI-1 complexes is obtained. uPA-PAI-1 complexes are expressed as uPA equivalent (i.e., 1 ng of uPA-PAI-1 complexes corresponds to 1 ng of uPA complexed with PAI-1). The exact uPA-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. **CII**: 1 vial containing 0.5 ml of lyophilised **Plasma Control II Low** (human plasma).

Note: The uPA-PAI-1 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**: One vial of 6 ml of **0.45M Sulfuric Acid** (**Stop solution**). Ready to use.

***Note*:** Use only components from a same kit lot. Do not mix components from different lots, 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. **uPA-PAI-1 Calibrator:** restore each vial with **2 ml of** F-Sample Diluent. 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:** 0.45M Sulfuric acid, 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.

The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

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, uPA-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 uPA-PAI-1 concentrations > 2C 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 **uPA-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.

uPA-PAI-1 concentration (ng/ml)	C	C/2	C/4	C/10	C/20	0 ng/ml
Vol. of uPA-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
uPA-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_2O_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. Calibrators, controls and tested specimen must be distributed as rapidly as possible on the microElisa plate (within 10 min.) in order to allow obtaining homogeneous immunological kinetics for immunocapture.

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. An incubation temperature of 18-25°C must be respected. Results are affected by a too high (>25°C) or too low (<18°C) temperature, and measured A450 are then too high or too low. It has to be considered when analyzing the results. In the same way, if a microplate shaker is used, it should be used only at the beginning of each step (sample introduction, immunoconjugate introduction, stop solution introduction), for 1 to 2 minutes, in order to obtain a good homogeneity. A450 values generated in the assay are significantly increased if shaking is used throughout the incubation steps.

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 ng/ml), the uPA-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, 50 µl of immunoconjugate (anti-(h)-PAI-1 peroxidase) are first introduced, then 200 µl 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 uPA-PAI-1 concentrations read must be multiplied by the sample dilution factor.

RESULTS:

Users must construct their own calibration curve, obtained using their standard dilutions.

- On a linear graph paper plot the **uPA-PAI-1 concentration** on abscissae and the corresponding absorbance on ordinates.
- From the curve obtained, deduce the uPA-PAI-1 concentration for the tested sample. For obtaining the uPA-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.

BIOCHEMISTRY:

Stoichiometric, stable, uPA-PAI-1 complexes are generated when an uPA molecule binds to its major inhibitor PAI-1. The complexes have a MW of about 100,000 daltons (55,000 for uPA and 50,000 for PAI-1), and are rapidly cleared from blood circulation. The concentration of uPA-PAI-1 complexes in normal human plasma is usually low (<5 ng/ml).