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.

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 immunocoujugate, which is an anti-tPA-1 monoclonal antibody coupled to horseradish 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. Cl: 1 vial containing 0.5 ml of lyophilised Plasma Control I (High) (human plasma).
5. CB: 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 immunocoujugate, 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.
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 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.

Nota: 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 (45%) 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 immunocoujugate: each vial must be restored with 7.5 ml of Conjugate Diluent. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogeneize the content. 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.

Nota: 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 ml) 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 twofold (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 twofold (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 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 homogenisation.

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 reagents, on the flyer provided in the kit, prepare the following standard solutions.

**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, 50 ml 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 TMS 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 concentrations on abscissa and the corresponding absorbances on ordinate.
- 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.

**Example of Calibration Curve:**

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

**Expected Range:**

- The concentration of tPA-PAI-1 complexes in normal human plasma is usually low (< 5 ng/ml).
- It increases with age, exercise and stress.

**Biochemistry:**

Stoichiometric, stable tPA-PAI-1 complexes are generated when an 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.

**Pathological Variations:**

Increased concentrations of tPA-PAI-1 complexes are usually observed in all clinical situations associated with elevated PAI-1 levels. There is then a reactive release of tPA from endothelial walls and complexes are then formed.

**Applications:**

- Assay of tPA-PAI-1 complexes in clinical samples, as a marker of disease.
- Assay of tPA-PAI-1 complexes in research studies (cell culture supernatants,...).

**References:**