

ZYMUTEST tPA Antigen

ARK011A-RUO

(Complete ELISA kit for Tissue-Type Plasminogen Activator Antigen)

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

ANIARA

Manufactured By: HYPHEN BioMed

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INTENDED USE:

The ZYMUTEST tPA kit is a two-site immuno-assay for measuring human Tissue-plasminogen Activator (tPA) in plasma. **This assay 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 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 colour develops. The amount of colour developed is directly proportional to the concentration of human tPA:Ag in the tested sample.

TEST SAMPLE:

Trisodium Citrate or Na₂ EDTA anticoagulated human plasma.

Note: This kit can be used to measure the tPA:Ag in biological fluids only if an appropriate standard is used.

REAGENTS:

- 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.
- SD:** 2 vials containing 50ml of **F-Sample Diluent**, ready to use.
- STD:** Four vials of 1ml, freeze-dried, **tPA Standard 0, 1, 2, 3 at different concentrations for calibration** (normal plasma checked with the NIBSC international standard). Each vial has to be restored with 1 ml of distilled water. The exact tPA-Ag concentration of these different levels of standards is indicated on the flyer provided in the kit.
- CI:** 1 vial containing **1 ml** of lyophilised **Plasma Control I High (UTA)** (human plasma).
- CL:** 1 vial containing **1 ml** of lyophilised **Plasma Control II Low (UTA)** (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.

- IC:** 3 vials of **Anti-(h)-tPA-HRP immunoconjugate**, a monoclonal antibody coupled to HRP, lyophilised.
- CD:** 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
- WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
- TMB:** 1 vial of 25 ml peroxidase substrate: **3,3',5,5' - Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
- SA:** 1 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 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.

- Micro ELISA plate:** open the plastic pouch and take off the required amounts 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).

- 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.
- tPA Standard 0, 1, 2, 3:** restore each vials with **1 ml** of distilled water. These solutions are stable for at least **8 hours** at room temperature.

Note: For a multiple use of the kit, prepare 3 aliquots of each level of standard after reconstitution. These aliquots are then stable 2 months, stored frozen.

- Plasma Control I (UTA)** (human plasma, high): restore with **1 ml** distilled water.
- Plasma Control II (UTA)** (human plasma, low): restore with **1 ml** distilled water.

Note:

When restored, tPA controls 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 tPA standard (3) and controls (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.

- Anti-(h)-tPA-HRP immunoconjugate:** 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 homogenize 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**.
- 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.
- 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.
- 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.
- 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. 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 8 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.

Tested plasma or controls:

The **plasma samples** and the **Controls I and II** are tested **undiluted** in the wells (after reconstitution).

For expected tPA concentrations > 20 ng/ml, plasma or samples can be tested with a dilution of **1:2, 1:5, 1:10, or more**, according to the expected tPA concentration. Dilutions of tested samples must be done in F- Sample Diluent (F-SD).

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Calibration:

The 4 levels of **tPA standard 0, 1, 2, 3** are reconstituted with 1ml of distilled water, then used **undiluted** directly into the wells of the plate. A calibration range from **0 to about 20ng/ml** tPA concentration is obtained.

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 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
F-Sample Diluent	100 µl	Introduce the F-SD in the micro ELISA plate wells.
tPA standards (1:1) or tested samples or controls or F-Sample Diluent (blank)	100 µl	Introduce the standard solutions, the controls 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 (b).
Conjugate (anti tPA monoclonal antibody coupled with peroxidase. Restored with 7.5 ml of Conjugate Diluent)	200 µl	Introduce the Anti-(h)-tPA- 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 (a).
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:

- The two fold dilutions in F-SD of the plasma samples, the controls or of the standards can be performed in test tubes. Then, 200µl of the 1:2 diluted samples would be added in the micro ELISA plate wells. Distribute calibrators, controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for tPA binding.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development.
- 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.
- 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.
- 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 using a "one step method". In this case, the calibration curve must be from 0 to about 10 ng/ml, the four level of tPA standards being reconstituted with only **1 ml** of distilled water and then diluted two fold (**1:2**) in F-Sample diluent (The calibrators are prepared as for the "2 step" method, but are two-fold diluted).

The immunoconjugate (**IC**) must be reconstituted with **2 ml** of Conjugate Diluent (**CD**).

Tested plasma must be assayed at a two fold (**1:2**) dilution or at higher dilutions in F-Sample Diluent (**SD**). In the microwell, the immunoconjugate (**IC**) is introduced (**50 µl**), followed by introduction of **200µl** of the calibration solution or the diluted plasma.

Following 1 hour incubation at room temperature and a washing step, **TMB** is introduced (200µl/well), colour is allowed developing for 5 min, and is then stopped with 50 µl of 0.45M sulfuric acid (**SA**). Draw the calibration curve as indicated in results (**but from 0 to about 10 ng/ml**). The tPA: Ag concentrations read must be multiplied by the sample dilution fact (i.e. 1:2, or the actual dilution factor).

EXPRESSION OF RESULTS:

- On a linear graph paper plot the tPA:Ag concentrations, in ng/ml, on abscissa and the corresponding absorbances on ordinates. Draw the calibration curve that best fits your data.
- Users must construct their own calibration curve, obtained using their standard solutions (see model on the flyer).
- From the curve obtained, deduce **directly** the tPA:Ag concentration of the **tested sample** and of the **Controls CI and CII**. For a diluted sample, multiply the obtained concentration by the applied dilution factor.
- 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:

- Tissue-Type Plasminogen Activator (tPA), is a 68 KDa protein, synthesised and secreted by endothelial cells. It initiates fibrinolysis by activating plasminogen to plasmin on 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.

ASSAY CHARACTERISTICS:

- Detection threshold ≤ 0.5 ng/ml.
- Intra-assay: 3-8%.
- Inter-assay: 5-10%.
- No significant interference of:
 - heparin up to 2 IU/ml
 - endogenous PAI-1 up to 100 ng/ml
- The kit allows measuring homogeneously tPA, whether its presentation is, free and active or complexed with its inhibitors.

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