ZYMUTEST tPA Antigen

Ref RK011A-RUO

ELISA kit for tPA Antigen

FOR RESEARCH USE ONLY.

DO NOT USE IN DIAGNOSTIC PROCEDURES

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English, last revision: 04-2017

INTENDED USE:

The ZYMUTEST tPA kit is a two-site immuno-assay for measuring human Tissue-plasminogen Activator (tPA) in plasma. This kit can be used to measure the tPA:Ag in biological fluids only if an appropriate standard is used.
The kit allows measuring homogeneously tPA, whether its presentation is, free and active or complexed with its inhibitors.
This kit is for research use only and must not be used for patient diagnosis or treatment.

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[1]. When present, this protein is captured onto the solid phase. Following a washing step, the immunoconjugate, which is a monoclonal antibody coupled to horseradish 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[2].

REAGENTS:

1. **COAT: Micro ELISA plate:** containing 12 strips of 8 wells, coated with a murine monoclonal antibody specific for human tPA, stabilised and packed in an aluminium pouch hermetically sealed in presence of a desiccant. Contains BSA.
2. **F-SD: F-Sample Diluent:** 2 vials containing 50 mL of **F-Sample Diluent**, ready to use. Contains BSA.
3. **STD: tPA Standard 0, 1, 2, 3:** 4 vials of 1 mL of tPA Standard 0, 1, 2, 3 **at different concentrations for calibration,** lyophilized (normal plasma checked with the NIBSC international standard), titrated in tPA antigen. Each vial has to be restored with 1 ml of distilled water. The exact tPA-Ag concentration of these different levels of calibrators is indicated on the flyer provided in the kit.
4. **CI: Plasma Control I High (UTA)**: 1 vial of 1 mL, lyophilised. After reconstitution with 1 mL of distilled water, the plasma control is ready to use (human plasma, control high). Contains BSA.
5. **CII**: **Plasma Control II Low (UTA)**: 1 vial of 1 mL, lyophilised. After reconstitution with 1 mL of distilled water, the plasma control is ready to use (human plasma, control low). Contains BSA.
6. **IC: Immunoconjugate (Anti-(h)-tPA-HRP immunoconjugate):** 3 vials of immuconjugate, lyophilized. Monoclonal antibody coupled to HRP. After reconstitution with 7.5 mL of diluent for immunoconjugate (CD), the immunoconjugate is ready to use. Contains BSA.
7. **CD: Conjugate Diluent:** 1 vial of 25 mL of diluent, ready to use. Contains BSA.
8. **WS: Wash Solution:** 1 vial of 50 mL of diluent, 20 fold concentrated.
9. **TMB: 3,3', 5,5' – Tetramethylbenzidine:** 1 vial of 25 mL of diluent, ready to use. Containing hydrogen peroxide.
10. **SA: 0.45M Sulfuric acid (Stop solution)**: 1 vial of 6 mL of diluent. Ready to use.

The exact concentration of controls and calibrators and the acceptable interval concentration for the controls are indicated on the flyer provide in the kit. For the assay, refer to the values provided on the flyer of the kit.

Reagents SD, CD and WS contain 0.05% Kathon CG and reagent SA contains sulfuric acid, see WARNINGS AND PRECAUTIONS.

WARNINGS AND PRECAUTIONS:

- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- A yellow color of TMB substrate indicates a contaminated substrate. Discard the vial and use a new one.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits. Do not mix reagents from different kit batches when performing an assay; they are optimized for each batch of kits.
- Handle the reagents with care to avoid contamination during use. If possible, avoid reagent evaporation during use by limiting the liquid-air exchange surface.
- To preserve reagent stability, seal the vials after use with their respective caps.
- Aging studies, conducted over a 3-week period at 30°C, show that the reagents can be shipped at room temperature over a short period of time, without degradation.
- The human plasma used to prepare the calibrator and controls I and II has been tested by recorded methods and is certified free of HIV antibodies, Hbs Antigen and HCV antibodies.
- The bovine plasma used to prepare the BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Wear protection glasses and gloves when handling. Avoid any skin and eye contact.
- For *in vitro* use.

SA: H290 : May be corrosive to metals.
CD/SD/WS: H317 : May cause an allergic skin reaction.

REAGENT PREPARATION AND STABILITY:

Bring the kit at room temperature, at least 30 min before use. Store the unused reagents at 2-8°C. Vials are closed under vacuum. Remove carefully the stopper of lyophilized reagents, in order to avoid any loss of powder when opening the vials.
When appropriately used and stored, according to the recommended protocol and cautions, the kit can be used over a 1 month period, and strip by strip, if required.

1. **COAT (Micro ELISA plate):** Open the aluminium 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 plastic microplate storage bag (minigrip).
2. **F-SD (F-Sample Diluent):** Ready to use. This reagent contains 0.05% Kathon CG.
   Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **4 weeks** at 2-8°C.
3. **STD (tPA Standard 0, 1, 2, 3):** Reconstitute each vial with exactly **1 mL** of distilled water in order to constitute a calibration range titrated in tPA.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
   - **8 hours** at room temperature (18-25°C).

   After reconstitution, prepare aliquots for each level of standard in 3 vials for fractional use. Stability of aliquots is:
   - **2 months** frozen at -20°C or below.
4. **CI (Plasma Control I (human plasma, control high, UTA):** Reconstitute each vial with **1 mL** of distilled water.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **8 hours** at room temperature (18-25°C).
   - **24 hours** at 2-8°C.
   - **2 months** frozen at -20°C or below.
5. **CII (Plasma Control II (human plasma, control low, UTA):** Reconstitute each vial with **1 mL** of distilled water.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **8 hours** at room temperature (18-25°C).
   - **24 hours** at 2-8°C.
   - **2 months** frozen at -20°C or below.
6. **IC (Anti-(h)-tPA-HRP immunoconjugate):** Reconstitute each vial of immunoconjugate with exactly **7.5 mL of "Conjugate Diluent"** at least 15 min before use. Let the pellet to be completely dissolved before use, and shake the vial in order to homogenize the content.
   Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **24 hours** at room temperature (18-25°C).
   - **4 weeks** at 2-8°C.
7. **CD (Conjugate Diluent):** Ready to use. This reagent contains 0.05% Kathon CG. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C.
8. **WS (Wash Solution):** Incubate, if necessary, the vial in a water bath, at **37°C**, until complete dissolution of crystals. Shake the vial and dilute the amount required **1:20** in distilled water (the 50 mL contained in the vial allow to prepare 1 liter of Wash Solution).
   Stability of the wash solution, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C.

   Stability of the dilute wash solution, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **7 days** at 2-8°C.

   This reagent contains 0.05% Kathon CG.
9. **TMB:** Ready to use. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
   - **4 weeks** at 2-8°C.
10. **SA (Stop Solution):** Stop solution containing 0.45M sulfuric acid, ready to use. See CAUTIONS AND WARNINGS. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial:
    - **4 weeks** at 2-8°C.

STORAGE CONDITIONS:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.

Materials:

- **8-channel pipettes** allowing dispensing volumes of 50-300 µL**.**
- **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.

SPECIMEN COLLECTION AND PREPARATION:

Specimens should be prepared and stored in accordance with applicable local guidelines.

- Specimens:

Human plasma obtained from anticoagulated blood (trisodium citrate). EDTA collected human plasma may also be used. The storage conditions are the same with citrated plasma.

- Collection:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M) by clean venipuncture. Discard the first tube.

- Centrifugation:

Within 2 hours, use a laboratory-validated method to obtain platelet-poor plasma, for example at least 15 minutes at 2500g at room temperature (18-25°C) and allow the plasma to settle in a plastic tube.

- Plasma storage:
  - 24 hours at room temperature (18-25°C).
  - 6 months at -20°C.

Frozen plasma specimens should be thawed rapidly at 37°C, then shaken thoroughly and tested immediately. Resuspend any precipitate by shaking vigorously immediately after thawing and before use.

PROCEDURE:

Assay procedure:

1. 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).

2. 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.

3. Remove the required number of strips from the aluminium pouch and 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 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.
Incubate for 1 hour at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings using the washing instrument **(a).**
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 **(b)**.
Incubate for 1 hour at room temperature (18-25°C) (a)		
Wash Solution (20 fold diluted in distilled water)	**300 µL**	Proceed to 5 successive washings using the washing instrument**.**
TMB/H_2O_2 Substrate	**200 µL**	Immediately after the washing, introduce the substrate into the wells. **Nota:** The substrate distribution, row by row, must be accurate and at exact time intervals **(b, c)**.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (a)		
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) (d)**. Subtract the blank values		

Remarks:

a) Distribute calibrators, controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for tPA binding.

b) 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.

c) 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.

d) 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 tPA standards (**Std 0, Std 1, Std 2, Std 3**) 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 (200 µL/well), colour is allowed developing for 5 min, and is then stopped with 50 µL of 0.45M sulfuric acid (**SA**). Do are measured at 450 nm and the calibration curve is drawn as indicated in results section (**but from 0 to about 10 ng/mL**). The tPA: Ag concentrations read must be multiplied by the sample dilution fact.

RESULTS:

- Results are expressed with **OD_{450}** obtained for the sample and control using the calibration curve.
- Plot the calibration curve, with the **OD 405 nm** along the Y-axis and the tPA Ag concentration, expressed as ng/mL, along the X-axis by choosing the "best fit" interpolation mode (refer to the flyer in the kit).
- 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 use only and must not be used for patient diagnosis or treatment.

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully. The laboratory is responsible for validating any changes made to these instructions for use.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- Any plasma displaying a coagulum or showing signs of contamination must be rejected.

PERFORMANCE:

Detection threshold ≤ 0.5 ng/mL.
Intra-assay: 3-8%.
Inter-assay: 5-10%.
No significant interference of heparin up to 2 IU/mL and endogeneous PAI-1 up to 100 ng/mL.

REFERENCES:

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

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.