ZYMUPHEN tPA Activity
Ref 521296

Microplate bio-immunoassay for the measurement of tPA Activity.
FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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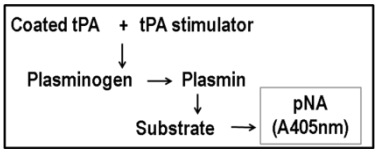
INTENDED USE:
Zymuphen tPA Activity kit is a bio-immunoassay for measuring tPA activity in human acidified citrated plasma or in purified milieu, using a microplate.
This kit is for research use only and should not be used for patient diagnosis or treatment.

SUMMARY AND EXPLANATION:
Tissue-Type Plasminogen Activator (tPA), is a 68 KDa protein, synthesized 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. tPA activity in plasma of healthy individuals is of about 0.5 IU/mL[1].

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, and that does not react with the tPA active site. When present, tPA is captured onto the solid phase. Following a washing step, in the presence of tPA stimulator (R2) that enhances the reaction, plasminogen (R2) is then converted into plasmin. Plasmin then reacts with its specific substrate (R1) and free pNA is released. tPA activity in the sample is directly proportional to the amount of free pNA measured at 405nm with a spectrophotometer.



Specimens:
Citrate 0.5M, pH 4.30 anticoagulated human plasma (see paragraph "specimen collection").
Any biological fluid or milieu where tPA activity must be measured.

REAGENTS:
1. **COAT: 12x 8-well strips microplate**, coated with a highly purified murine monoclonal antibody specific for human tPA, then stabilized; the plate is packed in an aluminum pouch hermetically sealed in presence of a desiccant.
2. **SD-CIT:** 2 vials containing 50mL of **Citrate-Phosphate Sample Diluent**, ready to use.
3. **R1:** 2 vials of plasmin substrate CS 41(03), lyophilized.
4. **R2:** 2 vials of plasminogen reagent, containing tPA stimulator, lyophilized.
5. **CAL:** 2 vials containing **2 mL** of **tPA calibrator**, lyophilized.
6. **CI:** 2 vials containing **0.5 mL** of lyophilized **tPA Control I High**.
7. **CII**: 2 vials containing **0.5 mL** of lyophilized **tPA Control II Low**.
8. **WS:** 1 vial of 50 mL of 20 fold concentrated **Wash Solution**.
9. **CA:** 1 vial of 6 mL of 2% citric acid (**Stop Solution**).

The concentration for each control and calibrator is indicated on the flyer provided in the kit. The tPA concentrations for the controls and calibrators may slightly vary from lot to lot. For the assay, refer to the concentration indicated on the flyer provided in the kit used.
Reagent 2 contains low concentration of Sodium azide (0.9 g/L), see CAUTIONS AND WARNINGS

CAUTIONS AND WARNINGS:
- Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.
- Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.
- The disposal of waste materials must be carried out according to current local regulations
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay; they are optimized for each lot of kits.
- Reagents must be handled with care, in order to avoid any contamination during use. Take care to limit as much as possible any evaporation of the reagents during use, by limiting the liquid-air surface exchange. Evaporation reduces reagent stability on instrument board.
- In order to improve stability, reagents must be closed with their original screw cap following each use.
- Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

PREPARATION AND STABILITY OF REAGENTS:
Vials are closed under vacuum. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.
1. **Coated microplate:** 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).
2. **SD-CIT Sample Diluent:**
Ready to use. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial or in a closed plastic microcentrifuge tube:
   - **4 weeks** at 2-8°C.
3. **R1:** Reconstitute each vial with exactly **6 mL** of distilled water, shake thoroughly for complete homogenization, let the reagent stabilize for 15 min at room temperature (18-25°C); while shaking the vial from time to time.
Homogenize before each use.
Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial or in a closed plastic microcentrifuge tube:
   - **24 hours** at 2-8°C.
   - **8 hours** at room temperature (18-25 °C).
   - **2 months** frozen at -20°C or below*
4. **R2:** Reconstitute each vial with exactly **6 mL** of distilled water, shake thoroughly for complete homogenization, let the reagent stabilize for 15 min at room temperature (18-25°C); while shaking the vial from time to time.
Homogenize before each use.
Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial or in a closed plastic microcentrifuge tube:
   - **24 hours** at 2-8°C.
   - **8 hours** at room temperature (18-25 °C).
   - **2 months** frozen at -20°C or below*
5. **tPA Calibrator:** Reconstitute each vial with exactly **2 mL** of distilled water at least 15 minutes before use to obtain a ready to use calibrator containing an activity "**C**" (in IU/mL) of tPA. Shake thoroughly for complete homogenization.
Homogenize before each use.
Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial or in a closed plastic microcentrifuge tube:
   - **24 hours** at 2-8°C.
   - **8 hours** at room temperature (18-25 °C).
6. **tPA Control I (high):** Reconstitute each vial with exactly **0.5 mL** of distilled water at least 15 minutes before use. Shake thoroughly for complete homogenization. **Dilute 1:2 in SD-CIT for the test**.
7. **tPA Control I (low):** Reconstitute each vial with exactly **0.5 mL** of distilled water at least 15 minutes before use. Shake thoroughly for complete homogenization. **Dilute 1:2 in SD-CIT for the test**.
Stability of tPA Controls, provided that any contamination or evaporation is avoided, kept in its original vial or in a closed plastic microcentrifuge tube:
   - **24 hours** at 2-8°C.
   - **8 hours** at room temperature (18-25 °C).
   - **2 months** frozen at -20°C or below*
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. **Stop solution:** 2% citric acid solution, ready to use.

*Thaw once as rapidly as possible at 37°C, adapt duration to the volume of reagent. The stability of the thawed reagent should be verified in the working conditions of the user laboratory.

STORAGE CONDITIONS:
Unopened reagents must be stored at 2-8°C, in their original packaging box. They are then usable until the expiration date printed on the label.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:
Reagents:
- Distilled water.

Materials:
- Micro ELISA plate washing equipment (and shaker).
- Spectrophotometer or automatic instrument for microplate chromogenic assays, with a wave-length set up at 405 nm. (reading range up to 4u of OD)
- Stopwatch; Calibrated pipettes.
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SPECIMEN COLLECTION:

Preparation and storage of specimens must be performed according to the current local regulations.

- Specimens:

Human plasma obtained from acidic trisodium citrate anticoagulated blood.

- Collection:

Blood (9 vol.) must be collected on acidic trisodium citrate anticoagulant (1 vol.) (citrate 0.5M, pH 4.30, e.g. Biopool® Stabilyte™ tube) in order to avoid any tPA inactivation, through a net venipuncture. The first tube must be discarded.

- Centrifugation:

Within 2 hours, use a validated method in the laboratory to obtain a platelet-poor plasma, e.g., a minimum of 15 minutes at 2500 g at room temperature (18-25°C) and plasma must be decanted into a plastic tube.

- Storage of plasma[2]:
  - 4 hours at room temperature (18-25°C)
  - 1 month at -20°C.
  - 18 months at -70°C.

Frozen plasma specimens should be rapidly thawed at 37°C, then gently mixed and tested immediately. Resuspend any precipitation by thorough mixing immediately after thawing and before testing.

TEST PROCEDURE:

Assay method:

1. Calibrators should be diluted using sample diluent as described in the table below in order to establish the calibration range ("C" = defined tPA concentration):

Calibrator	C	C:2	C:4	C:10	C:20	0
Volume of calibrator	1 mL	0.5 mL	0.25 mL	0.1 mL	0.05 mL	0 mL
Volume of sample diluent	0 mL	0.5 mL	0.75 mL	0.9 mL	0.95 mL	1 mL

2. The samples should be diluted using SD-CIT sample diluent as described in the table below:

Sample	Dilution
Controls	**1:2**
Specimens	**1:2**

Run the calibration curve and test it with quality controls. Diluted sample should be tested within 1 hour when stored at room temperature (18-25°C).
Please note that the exact concentration of the calibrators and controls is indicated for each lot on the flyer provided with the kit.

3. Into the wells, introduce:

Reagents	Volume	Test procedure
Calibrators, or controls or samples diluted 1:2 in SD-CIT, or sample diluent (blank)	**200 µL**	Introduce **immediately** samples or calibrators or controls into the wells.
Incubate 1 hour at room temperature (18-25°C)(a).		
Wash solution (WS) (diluted 1 :20 in distilled water before use).	**300 µL**	Proceed to 5 successive washings using the washing instrument **(b)**.
R1 substrate pre-incubated about 20 min. at 37°C, and homogenized before use.	**100 µL**	Immediately after the washing, put the plate (on a water-bath) at 37°C and introduce the substrate into the wells.
R2 plasminogen pre-incubated about 20 min. at 37°C, and homogenized before use.	**100 µL**	Introduce the plasminogen reagent into the wells**.(c)**
Incubate exactly 30 minutes at 37°C (water-bath)		
Stop solution	**50 µL**	Following exactly the same time intervals as for the addition of substrate, stop the colour development by introducing the 2% citric acid. **(c).**
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **405 nm (A405) (d)**. Subtract the blank values (d).		

Nota:
Distribute calibrators, controls and tested specimen as rapidly as possible, in order to obtain a homogeneous immunological kinetics for tPA binding. A too long delay (>10 min) between the distribution of the first and the last wells may have incidence on immunological kinetics and produce inaccurate results (underestimated value for the last wells).

a. Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. An incubation temperature of 18-25°C must be respected. Results can be affected by a too high (>25°C) or too low (<18°C) temperature, and measured A405 could then be too high or too low. It has to be considered when analyzing the results. A405 values generated in the assay are susceptible to be significantly increased if shaking is used throughout the incubation steps.
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 immobilised 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 plasminogen R2, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction with sulphuric acid.
d. For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used

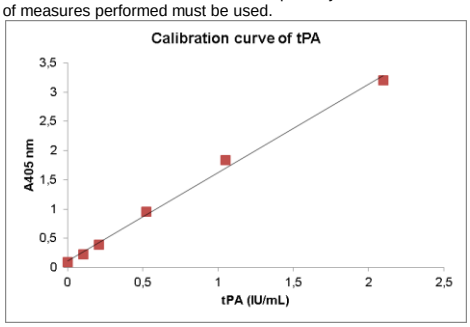
It is responsibility of the user to validate any modifications and their impact on all assay results.

CALIBRATION:

Using a linear scale:

- The assay is linear from 0 to about 2 IU/mL

The calibration curve below is indicated as an example only. The calibration curve generated for the series of measures performed must be used.



QUALITY CONTROL:

Using quality controls, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents.
Quality control must be included in each series, as per good laboratory practice, in order to validate generated results. A new calibration curve must be carried out preferentially for each test series, and at least for each new lot of reagents or, after each important analyzer's maintenance, or when quality controls values are measured outside the acceptance range determined for the method.
Each laboratory should establish and verify its own target values, acceptance ranges and expected performances, according to the instruments and protocols used.

RESULTS:

- For the end point manual method, using a linear graph paper plot, on abscissae, the tPA concentration (IU/mL) and on ordinates the corresponding absorbance (A405).
- From the curve obtained, deduce the tPA concentration for the tested sample and controls. For obtaining the tPA concentration, **the value read on the calibration curve must be multiplied by the dilution factor** (**i.e., x2 for controls and plasma diluted 1:2,** and xD for samples tested at 1:D dilution).
- Results are expressed in IU/mL.
- Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations. Choose the curve that best fits your data, as an example, it is possible to use a Lin-Lin linear regression. The tPA concentrations of CI and CII controls have been determined using a Lin-Lin linear regression. (The target values as well as acceptance ranges of controls must be verified in the exact laboratory working conditions and adjusted if required).

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

LIMITATIONS:

- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.
- Any reagent presenting an unusual aspect or a contamination sign must be rejected.
- Any plasma containing a coagulum or contamination must be rejected.

PERFORMANCES:

- The lower limit of detection is ≤ 0.1 IU/mL.
- The assay working range is from 0 to about 2 IU/mL (**0 to 4 IU/mL** in plasma before dilution 1:2).
- Recovery in plasma: 100% for the 1:2 dilution, 98% for undiluted plasma.
- Cross-reactivity: No significant cross-reactivity was observed with urokinase (uPA).
- The precision was performed in-house. Precision was evaluated with the quality control of the laboratory. Following data were obtained:

Controls	Intra assay		Inter assay	
	n	CV%	n	CV%
CI	12	7.7%	7	5.7%
CII	12	7.5%	7	8.6%

REFERENCES:

1. Bos R. et al, "Production and characterization of a set of monoclonal antibodies against Tissue-Type Plasminogen Activator (tPA)". Fibrinolysis, 6: 173-182, 1992.
2. Woodhams B, Girardot O, Blanco M-J, Colesse G, Gourmelin Y.Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001. Vol 12, No 4. 229-236.

SYMBOLS:

Used symbols and signs listed in the ISO standard 15223-1.