

ZYMUPHEN tPA Activity

Ref. 521296

Microplate bio-immunoassay for the measurement of tPA Activity

In vitro research use only

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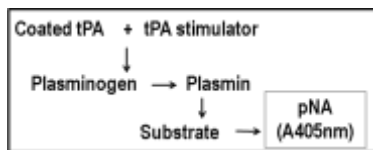
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INTENDED USE:

Zymuphen tPA Activity kit is a bio-immunoassay for measuring tPA activity in human acidified citrated plasma or in purified milieu, using a microplate.

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.



SPECIMEN:

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

REAGENTS:

- COAT:** 12x 8-well strips microplate, 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-CIT:** 2 vials containing 50ml of **Citrate-Phosphate Sample Diluent**, ready to use.
- R1:** 2 vials of plasmin substrate CS 41(03), lyophilised.
- R2:** 2 vials of plasminogen reagent, containing tPA stimulator, lyophilised.
- CAL:** 2 vials containing 2 ml of **tPA calibrator**, lyophilised.
- CI:** 2 vials containing 0.5 ml of lyophilised **tPA Control I High**.
- CII:** 2 vials containing 0.5 ml of lyophilised **tPA Control II Low**.

Nota: The tPA concentrations and acceptancy ranges for controls and calibrator can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.

- WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
- CA:** 1 vial of 6 ml of 2% citric acid (**Stop Solution**).

Nota: Use only components from a same kit lot. Do not mix components from different lots of kits, when running the assay.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

- Calibrated pipettes.
- Micro ELISA plate washing equipment (and shaker).
- Spectrophotometer, photometer or automates for microplate chromogenic assays, with a wave-length set up at 405 nm.
- Distilled water, preferentially sterile.
- Stop watch.

TRACEABILITY TO THE REFERENCE MATERIAL:

The concentration of tPA calibrator and controls is established for each lot against an internal standard. The internal standard is precisely determined against Tissue plasminogen activator, human, recombinant international standard from NIBSC, code 98/714.

STORAGE CONDITIONS:

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

Nota: 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:

- 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).
- SD-CIT 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.
- R1:** Reconstitute each vial with exactly **6 mL of distilled water**. Shake thoroughly until complete dissolution of the content (vortex). Let to homogenize for 15 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Homogenize the content before each use. Stability of restored reagent R1, provided that any contamination or evaporation is avoided, kept in its original vial: **24 hours at 2-8°C, 8 hours at room temperature (18-25 °C) and 2 months at -20°C or below**.
- R2:** Reconstitute each vial with exactly **6 mL of distilled water**. Shake thoroughly until complete dissolution of the content (vortex). Let to homogenize for 15 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Homogenize the content before each use. Stability of restored reagent R1, provided that any contamination or evaporation is avoided, kept in its original vial: **24 hours at 2-8°C, 8 hours at room temperature (18-25 °C) and 2 months at -20°C or below**.
- tPA Calibrator:** restore each vial with **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. Let the pellet to be completely dissolved before use, and shake the vial in order to homogenize the content (vortex). This solution is stable for at least **8 hours at room temperature (18-25°C) and 24 hours at (2-8°C)**.
- tPA Control I (high):** restore with **0.5 ml** distilled water at least 15 minutes before use, let the pellet to be completely dissolved before use, and shake the vial in order to homogenize the content (vortex). **Dilute 1:2 in SD-CIT for the test.**
- tPA Control II (low):** restore with **0.5 ml** distilled water at least 15 minutes before use, let the pellet to be completely dissolved before use, and shake the vial in order to homogenize the content (vortex). **Dilute 1:2 in SD-CIT for the test.**

Nota: when restored, undiluted 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: Plasminogen and tPA stimulator (R2) were prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. Bovine Serum Albumin (BSA) was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.

SD-CIT sample diluent contains low concentrations of **sodium azide** (0.9 g/l) that may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

- 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.
- Stop solution:** 2% citric acid solution, ready to use.

TESTED SPECIMEN:

Collection and preparation: Blood (9vol.) must be collected on citrate 0.5M pH 4.30 (1 vol.) (e.g.: Biopool® Stabilyte™ tube) in order to avoid tPA inactivation. Separate the plasma within 2 hours by centrifugation for 20 minutes at **3000 x g**.

Storage: The plasma must tested within 5 hours after collection, if stored at 5°C and is stable at -20°C or less for 6 months. Just before use, thaw the plasma during 15 minutes in a water-bath at 37°C.

Note: Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect....).

TEST PROCEDURE:

TESTED SAMPLES AND CONTROLS:

Samples and controls are assayed at the 1:2 dilution in Sample diluent (SD-CIT). The diluted samples must be tested within 1 hour.

CALIBRATION:

Using tPA Calibrator with a concentration "C" of tPA included in the kit, prepare the calibrators as follows:

Concentration	C	C:2	C:4	C:10	C:20	0
Vol of Calibrator	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol of sample diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

The dilutions are stable for 1 hour at room temperature (18-25°C).

ASSAY PROTOCOL:

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).

- 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.
- 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.
- 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.
- For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used

EXPRESSION OF RESULTS:

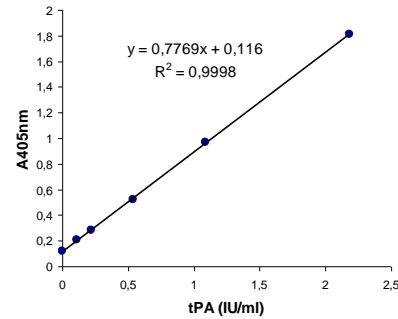
- On a linear graph paper, plot the tPA concentrations (IU/ml) on abscissa and the corresponding absorbances (A405) on ordinates. Draw the calibration curve that best fits your data (eg: lin-lin regression).
- Users must construct their own calibration curve, obtained using their calibrators (See the following model).
- 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).**

The calibration is validated when quality controls are measured within their acceptance range, indicated for each lot on the flyer provided in the kit.

- 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).

EXAMPLE OF CALIBRATION CURVE

This calibration curve is given as an example only for a calibrator at about 2 IU/ml. Use only the curve obtained with your own calibrators for generating results.



PERFORMANCES AND CHARACTERISTICS:

- Working range:** from 0 to about 2 IU/ml (0 to 4 IU/ml in plasma before dilution 1:2)
- Detection threshold:** ≤ 0.1 IU/ml.
- Intra-assay reproducibility (N= 12):** CV = 7.7% for CI, CV = 7.5% for CII.
- Inter-assay repeatability (N= 7) :** CV = 5.7% for CI, CV = 8.6% for CII.
- Recovery in plasma:** 100% for the 1:2 dilution, 98% for undiluted plasma.
- Cross-reactivity:** No significant cross-reactivity was observed with urokinase (uPA).

NORMAL RANGE

tPA activity in plasma of healthy individuals is of about 0.5 IU/ml (4). Using Zymuphen tPA Activity kit, the mean activity in normal plasmas collected with Biopool® Stabilyte™ tubes is of 0.6 IU/ml (N=13 healthy individuals), with concentrations ranging from 0.3 to 1.2 IU/ml.

PATHOLOGICAL VARIATIONS:

- Elevated concentrations of tPA are observed in various pathological conditions (respiratory distress syndrome, myocardial infarction, septicaemia, stroke, liver diseases, etc...). During liver transplantation, tPA concentrations are dramatically increased in the anhepatic phase.
- Recent epidemiological studies have demonstrated an association between tPA:Ag concentrations and an increased risk of cardiovascular diseases. Elevated tPA:Ag is frequently associated with high PAI-1 concentrations and a decreased basic fibrinolytic potential. Assay of tPA is then of predictive value for cardiovascular pathology and disease evolution.
- tPA is also involved in metastasis and tumor development, and as a prognosis marker of breast malignancy (3).

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.

APPLICATIONS:

- Assay of tPA activity in clinical samples, as a disease marker.
- Assay of tPA activity in thrombolysis with recombinant tPA drugs.

REFERENCES:

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