Tissue plasminogen activator (t-PA) is a 70,000 dalton glycoprotein which serves as the major activator of the blood fibrinolytic system. It is synthesized principally by endothelial cells and released into the blood stream following stimulus, e.g. exercise or venous occlusion. t-PA is unique among the plasminogen activators due to the fact that its action is highly fibrin specific. Only in the presence of fibrin, to which it binds, does t-PA efficiently activate Glu-plasminogen to yield the cleavage product, plasmin. Plasmin, in turn, degrades fibrin, the insoluble protein polymer in thrombi.t-PA circulates in plasma at a concentration of approximately 2-8 ng/mL. However, 95 % of circulating t-PA is complexed with the plasminogen activator inhibitor, PAI-1, and thus in an inactive form. Upon venous occlusion the concentration of t-PA antigen increases to 15 ng/mL or higher and t-PA's activity rises accordingly. This increase is probably due to a combination of increased t-PA release by endothelial cells and a reduction in t-PA clearance from the occlusion site.t-PA activity can only be measured in samples from which inhibitors of t-PA have been removed.In the Actibind system this is simply accomplished by specific immunoabsorption of the t-PA contained in the plasma sample by an antibody immobilized onto a microtitre plate surface. In this first step t-PA and t-PA-PAI-1-complexes are bound to the plate and active, non-complexed t-PA is therafter quantified using a solution of Glu-plasminogen, fibrinogen fragments and a synthetic substrate which reacts with plasmin formed in the reaction to yield a coloured reaction product. Low activity in this first step can be due to either a decrease in the quantity of total t-PA bound to the plate or to an excess of PAI-1 in the plasma sample, which inactivates t-PA and leads to the formation of t-PA-PAI-1-complexes. To differentiate between these two reasons for decreased t-PA function, the second part of the Actibind assay is then used to determine the total amount of t-PA antigen bound onto the microtiter plate through the use of a peroxidase-labelled monoclonal antibody.Normal plasma t-PA antigen values (2-8 ng/mL) are at the lower range of measurability making it difficult to determine significant reductions in plasma t-PA antigen levels. The activity measured in normal samples is also expected to be almost zero. To measure t-PA activity, venous occlusion plasma samples should be used (see test samples). The results obtained using the Actibind t-PA assay allow for the determination and clarification of various alterations in the fibrinolytic potential.

1. Increase in both t-PA antigen and activity.
This situation reflects an increase in t-PA release which is not counterbalanced by a simultaneous increase in PAI-1. This indicates a primary hyperfibrinolysis and likely will result in plasmin formation, consumption of plasmin inhibitors and degradation of other plasma proteins such as fibrinogen and factor VIII.

2. Increase in t-PA antigen with normal t-PA activity.
This situation reflects a continuous accumulation of t-PA due to decreased degradation by the liver which may occure in several hepatic disorders. When increased antigen levels are counteracted by a simultaneous increase in PAI-1, only t-PA antigen elevation is seen. Additionally, certain types of heparin therapy may result in significantly elevated t-PA antigen levels with low or insignificant increases in t-PA activity.

3. Decreased t-PA antigen and activity.
This situation can only be accurately determined by testing plasma samples drawn with venous occlusion. These plasmas should display elevations in both antigen and activity. When both t-PA activity and antigen are low in such plasma samples, this indicates a failure by the sample to respond appropriately to this type of stimulus. Approximately 10 % of all samples suffering from recurrent thrombosis fall within this group.

4. Decrease in t-PA activity with normal t-PA antigen.
Again, this situation can only be determined by testing plasma samples with expected high t-PA activity and antigen values, e.g. venous occlusion. In contrast to the group described in point 3, this group of samples shows a normal release of t-PA antigen in response to venous occlusion or other stimuli, but has such high PAI-1-levels that the released t-PA activity is counteracted by PAI-1 resulting in the formation of high levels of t-PA- PAI-1 complexes. Approximately 20 % of samples with thrombophilia fall within this group.

Application

The Actibind t-PA assay is specifically designed for the determination of t-PA antigen and activity in the same plasma sample.

Test principle

In the Actibind t-PA test an antibody which does not interfere with t-PA functional activity is coated onto a microtitre plate and used to bind t-PA contained in plasma to the plate surface. Following an incubation period, non-bound plasma components are washed away and an activity substrate solution containing Glu-plasminogen, CNBr-fragments of fibrinogen and a chromogenic plasmin substrate is incubated in the plate. The bound t-PA activates Glu-plasminogen to yield plasmin. The reaction between plasmin and the chromogenic plasmin substrate releases a coloured product whose concentration is proportional to the amount of active t-PA in the test sample. After photometrically measuring this reaction, the microtitre plate is washed to remove the activity substrate solution.The t-PA antigen remains bound to the plate. A horseradish peroxidase (POX) conjugated monoclonal anti-t-PA antibody which recognizes both active, t-PA and inactive t-PA is then incubated on the plate.
Following incubation and washing of the plate, a POX substrate is used to produce a coloured reaction product whose concentration is proportional to the total t-PA content of the test sample.

Specificity

The TC Actibind t-PA test measures t-PA functional activity, non-complexed t-PA antigen and t-PA-PAI-1 complexes. It is not affected by the presence of other plasminogen activators.
The measurement range is 0.05-10 IU/mL for t-PA activity and 0.1-20 ng/mL for t-PA antigen.
The inter- and intra-assay variations are less than 10 % and 5 %, respectively.

Test samples

Acidified citrate plasma (final pH =6) or EDTA plasma samples can be used. Commercially available tubes can be used. After filling, samples should be gently mixed by inverting the tube 5 times, and, then are placed in a crushed ice-water mixture. Centrifuge the blood within 90 minutes after the puncture at 2000 g for 30minutes at 4° C.
Pipette the pooled plasma into aliquots and store at a temperature below -30° C.
Thawing and refreezing of plasma aliquots is not recommended. Haemolytic and lipaemic plasmas may be used. In no case may plasma samples be used if any evidence of coagulation is seen.
Venous occlusion samples are obtained by applying a tourniquet around the upper arm with the pressure between the systolic and diastolic blood pressure, e.g. 100mm Hg, for at least 10 minutes. Draw blood from the arm before the pressure is reduced.

Kit Components

Determination: 42 samples in duplicate

1. **PLATE + PLATE COVER**
   12 x 8 well plastic microtitre strips precoated with a monoclonal anti-t-PA coating antibody in bicarbonate buffer, 1% bovine serum albumin (BSA), (TC-Code GN).
2. **STANDARD**
   1 x lyophilized recombinant t-PA, calibrated against NIBSC 86/670 (TC-Code AW).
3. **POX-ANTIBODY**
   1 x conjugated anti t-PA antibody (concentrated), 0,3ml .
   (TC-Code KM).
4. **INCUBATION BUFFER**
   1x90 ml (PBS; pH 7,3) contains stabiliser protein, 0,05% proclin and blue dye, 1bottle, , ready for use (TC-Code NB)
5. **SUBSTRATE - (green cap)**
   1x 12 mL TMB (tetramethylbenzidine) (TC-Code KN) ready to use.
6. **STOP SOLUTION - (red cap)**
   1x 15 mL sulphuric acid 0.45 mol/l (TC-Code KK) ready to use.
7. **WASHING BUFFER – concentrate (1+11,5)**
   1x80ml (PBS pH 7,3) containing detergent, 0,01 % merthiolat (TC-Code NA).
8. **PLASMINOGEN ACTIVATOR DETECTION MIXTURE.**
   1 x lyophilized H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide diacetate salt, Gluplasminogen and Cyanogen bromide fibrinogen fragments (TC Code BA).
9. **PLASMIN-ACTIVATOR DETECTION**
   MIXTURE DILUTION BUFFER (white cap)
   1 x 20 mL 50mM Tris, 12mM NaCl. Ready to use (TC-Code DA).

Kit storage: Store all components at 2 ... 8°C.

Also required

1. Micropipettes and a multichannel micropipette; pipette tips.
2. Glass or plastic test tubes for diluting the standard + samples.
3. Laboratory bottles or beakers and graduated cylinders for diluting wash and incubation buffer.
4. Distilled or deionised water.
5. Absorbent paper towels.
6. Microtitre plate washer (alternatively, washing can be performed manually using a multichannel pipette or repeating syringe).
7. A microtitre reader equipped with a 405 nm filter and, if possible, a 492 nm reference filter.
8. A 37° C incubator
9. Graph paper.

Reagent handling & bench stability

All reagents must be at ambient temperature before use.

Material/Reagent	State	Storage	Stability
Calibrator	after reconstitution	-20 °C	6 months
ELISA test strip	after opening	2 ... 8 °C with adhesive film in plastic bag with drying agent	expiry date
Washing buffer concentrate	after opening	2 ...8°C	6 months
Washing buffer	1+11.5 dilution of concentrate	2 ... 8 °C	3 weeks
Incubation buffer	after opening	2 ... 8 °C	2 months
Conjugate	after opening	2 ... 8 °C	6 months
	working solution	room temperature	60 minutes
Substrate TMB	after opening	2 ... 8 °C	expiry date

RT = Room Temperature
POX AB = Peroxidase conjugated Antibody

PLASMINOGEN ACTIVATOR DECTECTION MIXTURE RECONSTITUTION

a) Reconstitute the lyophilised plasminogen activator detection mixture (BA) in a total volume of 20mL by adding 5 mL of detection mixture dilution buffer (DA) to the vial.
b) Allow vial to stand at room temperature for 30 minutes then vortex well. Now mix the reconstituted detextion mixture with the 15mL dilution buffer (DA).

Stability of reconstituted mixture
2 hours at room temperature or aliquoted at –70°C for 2 weeks.

PLATE: Unused strips reseal in aluminium foil bag . Store at 4° C for up to 8 weeks.

STANDARD: Reconstitute the Standard with 0.5 mL aqua dest.

STANDARD CURVE for RAW11B4

	Dilution	Incubation Buffer	Antigen ng/mL	Activity IU/mL
A	0.155 mL Std.	0.425 mL	20	10
B	0.3 mL from A	0.3 mL	10	5
C	0.3 mL from B	0.3 mL	5	2.5
D	0.3 mL from C	0.3 mL	2.5	1.25
E	0 mL	0.3 mL	0	0

SAMPLE DILUTIONS
Dilute the plasma sample 1:4 with incubation buffer
100 µL plasma + 300 µL incubation buffer
Abnormally high plasma (i.e. samples collected during t-PA therapy or following venous occlusion) dilute 1:8
50 µL plasma + 350 µL incubation buffer

POX-CONJUGATED ANTIBODY
Preparing the conjugate working solution (1+50): Dilute 1 part by volume conjugate with 50 parts by volume incubation buffer.

For 8 test wells: Mix 20µl conjugate with 1000 µl incubation buffer.

ASSAY PROCEDURE

ACTIVITY ASSAY (405 nm)

1. SAMPLE/ STANDARD ADDITION
Pipette 0.1 mL of the diluted samples/standard into separate wells. Running sample/standard in duplicate is recommended.

2. SAMPLE INCUBATION
Cover the plate with a plastic foil and incubate overnight at 4°C.

3. WASH PLATE
Add 0.2 mL of wash buffer to the wells and tip out the contents. Wash the strips twice further with wash buffer. Tap strips on absorbant paper and make sure the wells are completely dry.

4. DETECTION MIXTURE ADDITION
Pipette 0.2 mL of the restored plasminogen activator detection mixture into each well, preferably with a multichannel pipette.

5. DETECTION MIXTURE INCUBATION
Cover the plate with a plastic foil and incubate for 90 minutes at 37° C.

6. READ: (FOR ACTIVITY CURVE)
Measure the absorbances at 405 nm (with 492 nm reference filter, if available)

7. GRAPH FOR ACTIVITY CURVE (A)
Construct activity curve using a point to point curve fit. Locate the absorbance for each sample on the curve and read the corresponding value from the horizontal axia. Do not forget to multiply by the dilution factor (4) or (8) for the samples.

Example of activity curve.
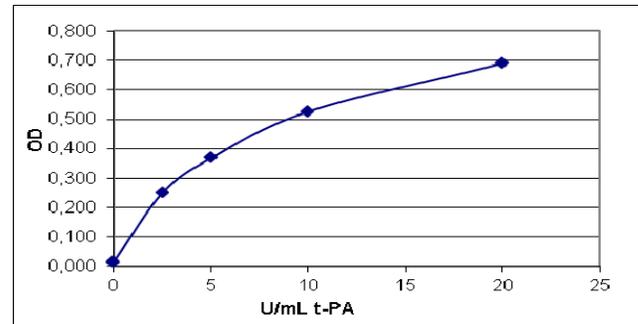
ANTIGEN ASSAY (450 nm)

8. WASH PLATE
Wash three times as described in step 3

9. POX ANTIBODY ADDITION
Add 0.1 mL of the diluted POX anti-t-PA antibody to all wells, preferably with a multichannel pipette.

10. POX ANTIBODY INCUBATION
Cover and incubate the plate for 1 hour at 37° C.

11. WASH PLATE
Wash three times as described in step 3.

12. SUBSTRATE
Pipette 0.1 mL of TMB substrate to all wells. Incubate for 10 minutes at room temperature.
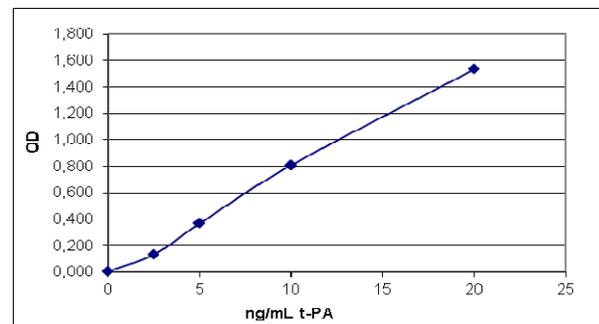
13. STOP
Pipette 0.1 mL of stop solution to all wells.

14. READ (FOR ANTIGEN STANDARD CURVE) (B)
Measure absorbances at 450nm (with 620nm reference filter if available). Read absorbances within one hour after the addition of the stop solution.

Example of Antigen standard curve.

15. GRAPH FOR ANTIGEN STANDARD CURVE

Construct a graph of standard curve using a point to point curve fit. Locate the absorbance for each sample on the curve and read the corresponding value from the horizontal axis. <u>Do not forget to multiply by the dilution factor (4) or (8) for the samples</u>.

Time table Summary of procedure		*time required*	*Temp.*
Reagent, standard sample handling		1-2 hours	
1. Sample – incubation	100 µL	overnight	4° C
wash 3 times	200 µL		
2. Detection Mixture – incubation	200 µL	90 minutes	37° C
Read absorbances 405nm			
wash 3 times	200 µL		
3. POX AB – incubation	100 µL	1 hour	37° C
wash 3 times	200 µL		
4. Substrate – incubation	100 µL	10 minutes	RT
Stop solution	100 µL		
Read absorbances 450 nm			
Time total: min.		**4 hours+ overnight**	

Evaluation of Results

Normal plasma levels: Antigen from 2-8 ng/mL, Activity approx. 0 IU/mL. For plasma obtained after venous occlusion values for Antigen greater than +15 ng/mL, for Activity +0.5 IU/mL should be obtained. A lack of demonstrable t-PA activity in plasma drawn with venous stasis is either indicative for a massive increase in plasma PAI-1 or of a defect in the sample's ability to synthesize or release t-PA. If, in the t-PA antigen part of the test, antigen measured in the same sample does not exceed 10 ng/mL after venous stasis, the lack of t-PA activity can be attributed to a lack of t-PA antigen synthesis and/or release. If, however, the t-PA antigen exceeds 10 ng/mL, an increased PAI-1 level is most likely to be the contributing cause of the reduced activity level. Both situations are indicative of a decreased fibrinolytic capacity and, thus, of an increased thrombotic tendency.

Appropriate measures should be taken to decrease the risk of thrombosis.

Increased basal t-PA antigen levels as well as the demonstration of active t-PA are indicative of hyperfibrinolysis.

For further analysis, the determination of other markers is recommended (PAP-complexes for hyperfibrinolysis, t-PA-PAI-complexes for liver function for which TC has available Kits: #12060 PAP complex ELISA and #12080 t-PA-PAI-1 complex ELISA).

Notes

Be sure to prepare all reagents before proceeding with the assay. It is critical to keep the time necessary for pipetting standards and samples to a minimum and avoid delays. Be sure to wash the plate thoroughly and completely remove any residual wash buffer after each wash cycle. Insufficient washing can lead to erroneously high values and incomplete removal of wash buffer to irregularities due to the dilution of added reagents.

As mentioned use a multichannel pipette to add peroxidase conjugate, TMB substrate and stop solution.

Warning

Potentially biohazardous material. Donor plasma used in this kit was tested by internationally approved methods for the presence of antibodies to HIV and hepatitis B virus and found to be negative. However, all human blood products should be handled as potentially infectious material.

Literature

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