TECHNOZYM® u-PA Combi Actibind ELISA Kit

For research use only REF TC16010

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Human urokinase (u-PA) is an enzyme which functions as an activator of the fibrinolytic enzyme system. Its ability to lyse fibrin clots makes it useful as an effective thrombolytic agent in the management of a variety of clinical disease states including pulmonary embolism and localized thrombosis. Urokinase is synthesized by and released from numerous cell types including endothelial cells, kidney cells and macrophages. Several malignant tumors, especially those of the urogenital and gastrointestinal tracts, have been shown to produce increased quantities of urokinase.
Urokinase exists in three major forms: enzymatically inactive single chain urokinase or pro-urokinase (scu-PA), enzymatically active two chain urokinase (tcu-PA) and urokinase-inhibitor complexes. scu-PA circulates in plasma at a concentration of 1-2 ng/mL and is converted to tcu-PA in vivo by the action of plasmin and kallikrein. Each form of u-PA displays a different activity, different affinities for Glu-plasminogen, and different rates of inhibition by plasma protease inhibitors.
For therapeutic administration, pro-urokinase is generally preferred over other forms of this plasminogen activator. Pro-urokinase is not inhibited by plasminogen activator inhibitors and, although no form of urokinase is highly fibrin specific, pro-urokinase is more fibrin-oriented than other forms of u-PA. During thrombolytic therapy, pro-urokinase is converted to the active two chain urokinase and is then susceptible to inhibition.
The Actibind u-PA assay employs specific reagents which allow for the measurement of urokinase activity originating from both the active two chain form and the single chain form of the molecule. It does not detect inactive urokinase-inhibitor complexes. u-PA antigen, however, is detected in all three forms (scu-PA, tcu-PA and u-PA inhibitor complexes) by this assay system. This allows the quantification of urokinase inhibitors.

Applications

The Actibind u-PA test is specifically designed to monitor u-PA antigen and function during thrombolytic therapy with single or two chain-u-PA.

Test Principle

In the Actibind u-PA test a monoclonal antibody which does not block u-PA functional activity is coated onto a microtiter plate and used to bind u-PA contained in plasma to the plate surface. Following an incubation period, non-bound plasma components are washed away and a detection mixture containing Glu-plasminogen and a chromogenic plasmin substrate is incubated on the plate. The bound u-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 u-PA in the test sample. After photometrically measuring this reaction, the microtiter plate is washed to remove the activity substrate solution.
The u-PA antigen remains bound to the plate. A biotinylated anti u-PA detecting antibody which recognises both active u-PA and inactive u-PA and streptavidin-horseradish peroxidase is then incubated on the plate.
Following incubation and washing of the plate, TMB substrate is used to produce a coloured reaction product whose concentration is proportional to the total u-PA content of the test sample.

Specificity

The TECHNOZYM Actibind u-PA test measures u-PA functional activity, non-complexed u-PA antigen and u-PA-inhibitor complexes.
It is not affected by the presence of other plasminogen activators.
The test system measures u-PA antigen in a range from 0-10 ng/mL and u-PA activity from 0-1.0 IU/mL. The inter- and intra-assay variations are less than 10 % and 5 %, respectively.

Test samples

Use fresh EDTA plasma samples with inhibitors. Whole blood freshly drawn into an anticoagulant cocktail mixture containing proteinase inhibitors. The mixture should contain 50-200 mM buffered EDTA in 1:10 ratio to blood as anticoagulant with aprotinin and benzamidine as inhibitors, again in 1:10 ratio to blood, yielding the final concentrations 10 IU/mL and 20 mM respectively. The samples should be gently mixed by inverting the tube 5 times, and, then placed in a crushed ice-water mixture. Centrifuge the blood within 30 minutes after the puncture at 2000 g for 30 minutes at 4 °C (preferably in a centrifuge with a swingout rotor). Immediately after centrifugation, plasma should be carefully pipetted and collected in a plastic tube. After careful mixing of the plasma, precooled and prelabelled plastic tubes for storage are filled. The freezing operation is very important: snap-freezing, that means almost instantaneous freezing, is highly recommended. Storage temperature should be kept constantly below -30 °C, and preferably at -70 °C. The total time between blood collection and plasma freezing should not exceed 90 minutes. Thawing and refreezing of plasma aliquots is not recommended. Thawing for assay is achieved rapidly using a water bath at 37° C. After thawing, plasma samples are placed in a crushed ice-water mixture until analysis.
Haemolytic and lipemic plasma may be used. In no case may plasma samples be used if any evidence of coagulation is seen. If only citrate plasma is available for testing, EDTA must be added to a 5 mM end-concentration before testing (1.86 mg EDTA/mL plasma) with the inhibitors to an end concentration as described above.
As a test control you may use commercially available clotting-factor control plasma. Be sure to add EDTA if necessary.
Undiluted plasma samples are normally used for testing. If testing plasmas in which abnormally high u-PA values are expected (for example during u-PA therapy) samples may be diluted (e.g. 1:4) with the enclosed dilution buffer.

Anti-Coagulant Cocktail Mixture

1 part cocktail: 9 parts blood
200 mM EDTA
200 mM benzamidine
0.1 % Tween 80
5.0 % bovine serum albumin
100 KIU/mL aprotinin
In PBS. Adjust to pH 7.4

Kit Components

1. **PLATE + PLATE COVER**
   12 x 8 well plastic microtitre strips precoated with a monoclonal anti-u-PA coating antibody in bicarbonate buffer,1% bovine serum albumin (BSA), (TC-Code GU).
2. **STANDARD**
   1 x lyophilized urokinase, calibrated against the WHO International Standard for High Molecular Weight Urokinase
   Concentrations are lot-dependent; consult batch table.
3. **POX ANTIBODY** (brown cap)
   1x conjugated polyclonal anti u-PA antibody (concentrated)
4. **DILUTION BUFFER -** (white cap)
   1 x 20 mL 2.5x concentrated (PBS, 1 % BSA, 20 mM EDTA, 10 KIU/mL aprotinin, 20 mM Benzamidine) (TC-Code DC).
5. **POX DILUTION BUFFER -** (white cap)
   2 x 12 mL PBS, 1 % BSA Ready to use (TC-Code DD).
6. **SUBSTRATE - (green cap)**
   1 x 12 mL TMB (Tetramethylbenzidine) in substrate buffer containing H_2O_2. Ready to use (TC-Code KN).
7. **STOP SOLUTION -** (red cap)
   1 x 15 mL 0.5 M Sulphuric Acid (TC-Code KK). Ready to use
8. **WASH BUFFER – concentrate**
   1 x 80 ml (PBS pH 7.3) containing detergent, 0.01 % merthiolate
9. **PLASMINOGEN ACTIVATOR DETECTION MIXTURE**
   1 x lyophilized H-D-norleucyl-hexahydrotyrosyl lysine-p-nitroanilide diacetate salt and Gluplasminogen (TC Code BA).
10. **DETECTION MIXTURE DILUTION BUFFER** (white cap)
    1x 20 mL 50 mM Tris, 12 mM 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 dilution buffer
4. Distilled or deionised water
5. Absorbent paper towels
6. Microtiter plate washer (alternatively, washing can be performed manually using a multichannel pipette or repeating syringe)
7. A microtiter reader equipped with a 405 nm and a 450 nm filter and, if possible, a 492 nm and a 620 nm reference filter
8. A 37 °C incubator

Reagent handling & bench stability

Material/Reagent	State	Storage	Stability
calibrator	after reconstitution	-20 °C	6 months
ELISA test strips	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	Dilution 1+11.5 of concentrate	2 ... 8 °C	3 weeks
POX Dilution buffer & Dilution buffer	after opening	2 ... 8 °C	6 months
POX Ab	after opening	2 ... 8 °C	6 months
	working solution	room temperature	60 minutes
Substrate & Stop solutions	after opening	2 ... 8 °C	expiry date
Detection mixture	After reconstitution	-20 °C	2 months

Preparation of reagent

1. Before starting the test, all the required components are to be brought to room temperature.
2. Preparing the washing buffer: Dilute 1 part by volume washing buffer concentrate with 11.5 parts by volume distilled water (1+11.5). Mix well (Diluted washing buffer concentrate = washing buffer). There may be crystalline precipitations which will dissolve at 37 °C within 10 minutes.
3. Preparing the Dilution buffer: Dilute 1 part by volume dilution buffer concentrate with 1.5 parts by volume distilled water (1+1.5). Mix well
4. Reconstituting calibrator:
   The calibrator is reconstituted with 500 µl distilled water and mixed for 10 seconds after a reconstitution time of 15 minutes (vortex mixer). Reconstituted components are clear to slightly turbid.
5. Preparing the detection mixture: Reconstitute the detection mixture with 20ml of Detection Mixture Dilution buffer (ready to use).
6. POX Antibody: Preparing the working solution (1+50).

For 8 test wells: **20 µl POX Ab with 1000 µl POX dilution buffer.**

STANDARD CURVE

Preparation of calibrators for standard curve see batch table.

SAMPLE DILUTIONS

Use undiluted plasma for normal plasma.

Abnormally high plasma (i.e. samples collected during u-PA therapy) dilute

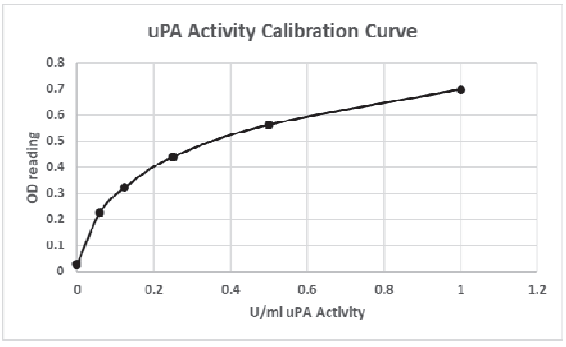
1:2 150 µl plasma + 150 µl dilution buffer

or 1:4 100 µL plasma + 300 µL dilution buffer

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	250 µL		
2. Detection Mixture-incubation	200 µL	70 minutes	37 °C
Read absorbances for activity curve at 405/492 nm			
Wash 3 times	250 µL		
3. POX Ab - incubation	100 µL	1 hour	37 °C
Wash 3 times	250 µL		
4. **Substrate - incubation**	100 µL	15 minutes	RT
Stop solution	100 µL		
Read absorbances for antigen standard curve 450/620 nm			

Assay Procedure

1. **SAMPLE/ STANDARD ADDITION**
   Pipette 0.1 mL of the diluted samples/standard into separate wells. Running standard/sample in duplicate is recommended.
2. **SAMPLE INCUBATION**
   cover the plate with a plastic foil and incubate overnight at 4 °C.
3. **WASH PLATE**
   Add 0.25 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 detection mixture into each well, preferably with a multichannel pipette.
5. **DETECTION MIXTURE INCUBATION**
   Cover the plate with a plastic foil and incubate for **70 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**
   Construct activity curve. 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 (2) or (4) for the samples.

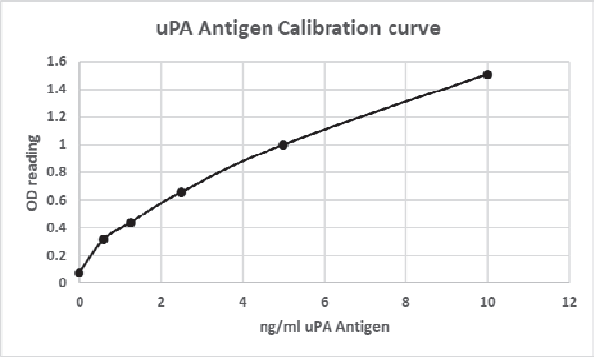


8. **WASH PLATE**
   Wash three times as described in step 3
9. **POX ANTIBODY ADDITION**
   Add 0.1 mL of the diluted POX antibody to each well, preferably with a multichannel pipette.
10. **POX ANTIBODY INCUBATION**
    Cover and incubate the plate for 1 hour at 37 °C.
11. **WASH PLATE**
    Wash three times as described in step 3.

12. SUBSTRATE
Pipette 0.1 mL of TMB substrate to each well. Incubate for 15 minutes at room temperature.

13. STOP
Pipette 0.1 mL of stop solution to all wells.

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



Construct a graph of standard curve. Locate the absorbance for each sample on the curve and read the corresponding value from the horizontal axis. Do not forget to multiply by the dilution factor (2) or (4) for the samples.

Evaluation of results

Plasma u-PA values within the normal range will be detectable at the lower end of the standard curve where exact values cannot be precisely determined. Clinical interest in u-PA centers on elevated u-PA levels. When testing plasma from patients undergoing u-PA therapy or those with pathological conditions leading to increased u-PA levels, it may be necessary to dilute the plasma with dilution buffer to obtain measurable results. Remember to multiply the u-PA value by the dilution factor.
u-PA plasma levels above 10 ng/mL, corresponding to 1 IU/mL can be caused by several malignant diseases. Further clarification is recommended

Literature

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