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Activity Assay for
Human tPA
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INTRODUCTION

Tissue-type Plasminogen Activator (tPA) is a member of the serine proteinase family. tPA functions to lyse fibrin clots into soluble plasmin fragments[1]. tPA is active in two forms, single chain and two-chain. The two-chain tPA is created via interaction with the plasmin product cleaving the single chain. This two-chain form is regarded as the more active form.

Both single chain and two-chain tPA are complexable with PAI-1. PAI-1 acts as an inhibitor for tPA by binding to the tPA and thus stifling its ability to lyse fibrin.

tPA can serve as an indicator of both myocardial infarction for patients with impaired fibrinolytic systems as well as a marker for type-II diabetes.

Clinical studies have indicated that high tPA levels may increase the risk for thrombosis[2], whereas decreased levels may cause neuronal plasticity and degeneration[3].

PRINCIPLES OF PROCEDURE

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of active tPA levels in biological fluid. This test kit operates on the basis of sandwich ELISA where free active tPA enzyme complexes with PAI-1 and is quantitated with the use of an HRP labeled secondary antibody.

First the biotinylated PAI-1 binds to the avidin coated wells. Next, active tPA present in the standard or unknown, complexes with PAI-1. Inactive or complexed tPA is removed in a subsequent wash step. A primary antibody specific for tPA is then added to each well followed by the HRP conjugated secondary antibody. The bound conjugated secondary antibody is detected by the addition of substrate, which generates an optimal color after 10 minutes. Quantitative test results may be obtained by the measure and comparison of the sample and standard absorbance readings when read with a microplate reader at 450 nm.

MATERIALS PROVIDED

Component	Contents	Quantity	Storage	Cat. No.
Coated Plate	Avidin coated 96-well plate	1 plate	4°C	PA90a
Standard	Human tPA activity standard (lyophilized)	1 vial	4°C	PA90b
Biotinylated PAI-1	Biotinylated PAI-1 (lyophilized)	1 vial	4°C	PA90c
Primary Antibody	Anti-human tPA monoclonal antibody (lyophilized)	1 vial	4°C	PA90d
Wash Buffer	10x solution for washing plate	50 mL	4°C	PA90e
Substrate	TMB Substrate	10 mL	4°C	PA90f
Secondary Antibody	Anti-rabbit HRP conjugated antibody	1 vial	4°C	PA90g
Assay Buffer	Buffer used to neutralize acidic samples	10 mL	4°C	PA90h

MATERIALS NEEDED BUT NOT PROVIDED

1. 1 N H_2SO_4

2. DI Water
3. Microplate reader with 450 nm filter
4. Microplate shaker with uniform horizontal circular movement up to 300 rpm
5. Precision pipettes that range from 10 µL-1000 µL and disposable tips

STORAGE

1. Store the kit and all of its components at 4°C before use.
2. If not using the entire plate at once, prepare only the appropriate amount of Primary Antibody and PAI-1 Standard. The remaining stock solutions should be frozen and stored at –70°C. Primary Antibody should be used within two weeks. All other components should remain refrigerated.
3. Store the reconstituted Biotinylated PAI-1 at 4°C. Use within two weeks.
4. Store unused portions of the microplate in a pouch with a desiccant at 4°C.

PROCEDURAL NOTES

1. This assay should be run at room temperature.
2. Use aseptic technique when opening and dispensing reagents.
3. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.
4. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

WARNINGS AND PRECAUTIONS

The tPA Standard is of human origin. Each donor unit has been tested and found negative for the presence of HBsAg, anti-HIV 1+2, anti-HBc, and anti-HCV.

Since no tests are currently available to assure that no infectious agents are present, the tPA Standard must be treated as is recommended at the Biosafety Level 2 as potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and Biomedical Laboratories", 1984.

SAMPLE COLLECTION, STORAGE, AND PREPARATION

Samples of human plasma in citrate or EDTA may be assayed with this kit. Plasma in heparin is not recommended. It is important to ensure a platelet free preparation as platelets can release PAI-1, which in turn could potentially form a complex with active tPA. Serum and cell culture media at neutral pH may also be used.

For best results, collect 9 volumes of blood in 1 volume of 0.1M acidified citrate, preferably using Stabilyte™ evacuated vials (Biopool, cat# 102080)[5]. The low pH of the resulting plasma insures that PAI-1 is inhibited from quenching tPA activity[6]. Immediately after collection of blood, samples must be centrifuged at 2500 x g for 15 minutes. The plasma must be transferred to a clean plastic tube and stored on ice prior to analysis. The tPA activity samples collected in the Stabilyte™ media are stable for up to 5 hours on ice, up to one month frozen at -20°C or up to 5 months frozen at -70°C. If sample pH is below 6.0 add 40 uL of assay buffer to all wells including the standard wells prior to adding samples to the plate.

REAGENT PREPARATION

1. **10x Wash Buffer:** Dilute the 50 mL of concentrate to 1x with 450 mL of DI water prior to use.
2. **TBS Buffer:** 0.1 M Tris, 0.15 M NaCl, pH 7.4.

3. **3% BSA Blocking Buffer:** 3% BSA in TBS Buffer.
4. **Biotinylated PAI-1:** Reconstitute with 10.0 mL of 3% BSA Blocking Buffer and vortex gently to mix. Prepare immediately prior to use.
5. **Standard:** Reconstitute with 10.0 mL of 3% BSA Blocking Buffer and vortex gently to mix. This will result in a 5.3 IU/mL standard solution. Use the 5.3 IU/mL working dilution to prepare all standards as outlined in the table below. Prepare immediately prior to use.
6. **Primary Antibody:** Reconstitute with 10.0 mL of 3% BSA Blocking Buffer and vortex gently to mix. Prepare immediately prior to use.
7. **Secondary Antibody:** Briefly centrifuge the vial prior to opening. Dilute 2.0 uL with 10.0 mL of 3% BSA Blocking Buffer and vortex gently to mix. Prepare immediately prior to use.

STANDARD PREPARATION

1. Reconstitute the standard by adding 10 mL of blocking buffer directly to the vial and agitate gently to completely dissolve the contents. The concentration will be 5.3 IU/mL.
2. Use the dilution table below to prepare all standards for the assay.

tPA Concentration (IU/mL)	uL of 5.3 IU/mL tPA Standard	uL of Blocking Buffer	Total Volume
1	100	430	530
.5	50	480	530
0.4	40	490	530
0.25	25	505	530
0.1	10	520	530
0.05	5	525	530
0.02	2	528	530
0.01	1	529	530
0	0	500	500

ASSAY PROCEDURE

1. Add 100 µl of reconstituted Biotinylated PAI-1 to all of the wells. Shake the plate at 300 rpm for 30 minutes at room temperature (RT).
2. Wash the plate 3 times according to the following wash procedure:
   a. Remove the contents of each well by inversion of the plate.
   b. Tap out the remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of 1x Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Repeat procedure two more times, then proceed to step “f”.
   f. Remove the contents of each well by inversion of plate into an appropriate disposal device.
   g. Tap out the remaining contents of the plate onto a lint free paper towel, then proceed to step 3.
3. Add 100 µl of the Standards and Samples to the wells in duplicate.
   NOTE: If the pH of the Samples is lower than 6.0, add 40 µL of the Assay Buffer to the wells prior to adding the Standards or Samples. This step is unnecessary if the pH is neutral.
   For a suggested plate layout, see Scheme I below. Shake the plate at 300 rpm for 30 minutes at RT.
4. Wash the plate three times as in step 2.
5. Add 100 µl of the Primary Antibody to each well. Shake the plate at 300 rpm for 30 minutes at RT.

6. Wash the plate three times as in step 2.
7. Add 100 µl of the Secondary Antibody to each well. Shake the plate at 300rpm for 30 minutes at RT.
8. Wash the plate three times as in step 2.
9. Add 100 µl of TMB Substrate to each well. Shake the plate at 300 rpm for 2-10 minutes at RT.
10. Stop the reaction by adding 50 µl of 1N H_2SO_4 to each well and read the plate at 450 nm.

Scheme I:

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_2
B	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	U_1	U_2
C	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}
D	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}
E	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
F	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}	U_{21}	U_{22}	U_{23}	U_{24}	U_{25}	U_{26}
G	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}
H	U_{27}	U_{28}	U_{29}	U_{30}	U_{31}	U_{32}	U_{33}	U_{34}	U_{35}	U_{36}	U_{37}	U_{38}

CALCULATIONS

1. Average the O.D. values for each pair of duplicate wells.
2. Plot the A_{450} against the concentration of uPA in the standards.
3. Fit a straight line through the points using a linear fit procedure.
4. Calculate the uPA concentrations in the unknowns using the standard curve.

EXPECTED VALUES

The basal level of tPA in healthy humans was found to be between 0.2-2 IU/mL[8].

Abnormalities in tPA levels have been reported in the following conditions:

- Neuronal plasticity and degeneration: Decreased levels of tPA have been implicated in the process of neuronal plasticity and degeneration[1,3].
- Arthritis: Decreased tPA levels may exacerbate arthritis[4].
- Deep venous thrombosis: Increased tPA levels may contribute to deep venous thrombosis[2].
- Coronary heart disease: Increased tPA levels may contribute to severe coronary heart disease[2].
- Pregnancy: Increased tPA levels are observed during pregnancy[7].

PERFORMANCE CHARACTERISTICS

Assay Range: 0 - 1 IU/mL (0 – 1.77 ng/mL)
Samples with tPA levels higher than 1 ng/mL should be diluted in similar media devoid of active tPA or 3% BSA Blocking Buffer.

Sensitivity: 0.006 IU/ml
(calculated by determining the OD of 20 reps of So and 20 reps of the low standard)

Intra Assay Precision

High: 3.8% Medium: 4.0% Low: 9.8%
(calculated by running 20 reps of each concentration in an assay)

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Oxford Biomedical Research, Inc.
P.O. Box 522
Oxford, MI 48371 U.S.A.
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