Activity Assay for
Mouse 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. 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.

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	PA92a
Standard	Mouse tPA activity standard	1 vial	4°C	PA92b
Biotinylated PAI-1	Biotinylated PAI-1 (lyophilized)	1 vial	4°C	PA92c
Primary Antibody	Anti-mouse tPA monoclonal antibody (lyophilized)	1 vial	4°C	PA92d
Wash Buffer	10x solution for washing plate	50 mL	4°C	PA92e
Substrate	TMB Substrate	10 mL	4°C	PA92f
Secondary Antibody	Anti-mouse HRP conjugated antibody	1 vial	4°C	PA92g
10x TBS Solution	Tris Buffered Saline pH 7.4	5 mL	4°C	PA92h

MATERIALS NEEDED BUT NOT PROVIDED

1. 1 N H_2SO_4
2. DI Water
3. Bovine Serum Albumin Fraction V (BSA)
4. Microplate reader with 450 nm filter
5. Microplate shaker with uniform horizontal circular movement up to 300 rpm
6. 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.

SAMPLE COLLECTION, STORAGE, AND PREPARATION

Samples should be collected using 0.1 M trisodium citrate or acidified citrate in a 1:10 ratio of collection media to blood. Immediately upon collection of blood, the samples should be centrifuged at 3000 x g for 15 minutes. This should ensure the removal of platelets as they can release PAI-1, that in turn complexes with tPA. The plasma should be transferred to a clean plastic tube to be kept on ice prior to analysis or stored frozen for up to one month. Samples are stable for approximately 24 hours when stored at 4°C or one month if stored at -20°C and thawed three times without loss of tPA activity.

Note: Detergents such as Triton X cause interference with the assay. If using detergent extracted samples, it is necessary to dialyze the samples overnight to remove the detergent.

REAGENT PREPARATION

1. **10x Wash Buffer:** Dilute the 50 mL of concentrate to 1x with 450 mL of DI water prior to use.
2. **10x TBS Buffer:** 0.1 M Tris, 0.15 M NaCl, pH 7.4. Dilute 5 mL of concentrate into 45 mL DI water.
3. **3% BSA Blocking Buffer:** 3% BSA in 1x TBS Buffer. Use 1.5 g BSA in 50 mL of 1x TBS.
4. **Biotinylated PAI-1:** Reconstitute with 3% BSA Blocking Buffer as directed on the vial and vortex gently to mix. Prepare immediately prior to use.
5. **Standard:** Reconstitute with 1.0 mL 3% BSA Blocking Buffer immediately prior to use and vortex gently to mix. The vial is now 1,000 ng/mL tPA. Prepare the standards according to the Dilution Table below.
6. **Primary Antibody:** Reconstitute with 10 mL of 3% BSA Blocking Buffer and vortex gently to mix. Prepare immediately prior to use.
7. **Secondary Antibody:** Dilute with 10 mL of 3% BSA Blocking Buffer and vortex gently to mix. Prepare immediately prior to use.

STANDARD PREPARATION

Standard	tPA Concentration (ng/mL)	Blocking Buffer (µL)	Transfer Volume (µL)	Transfer Source	Final Volume (µL)
S_{10}	50	950	50	Stock Vial	500
S_9	25	500	500	S_{10}	600
S_8	10	600	400	S_9	500
S_7	5	500	500	S_8	600
S_6	2	600	400	S_7	500
S_5	1	500	500	S_6	500
S_4	0.5	500	500	S_5	500
S_3	0.25	500	500	S_4	600
S_2	0.1	600	400	S_3	500
S_1	0.05	500	500	S_2	1,000
S_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 unknowns to the wells in duplicate.
   NOTE: If the pH of a sample is lower than 6.0, add 30 µL of the 10x TBS Solution to the well prior to adding the sample. 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 10-20 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.

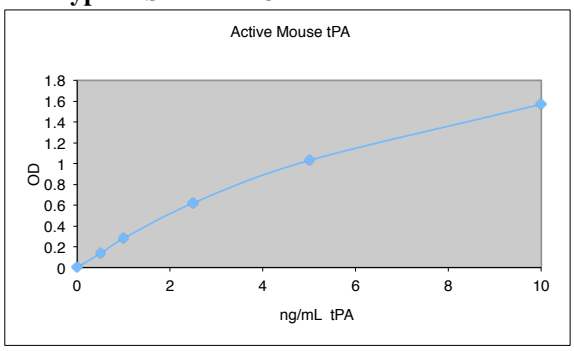
Scheme I:

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

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.

Typical Standard Curve:



PERFORMANCE CHARACTERISTICS

Assay Range: 0.1-50 ng/mL
Samples with tPA levels higher than 50 ng/mL should be diluted in similar media devoid of active tPA or 3% BSA Blocking Buffer.

REFERENCES

1. Thogersen A et al. (1998) Circulation 98: 2241-2247
2. Eliasson M et al. (2003) Cardiovascular Diabetology 2:19

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

Orders: 800-692-4633
Technical Service: 248-852-8815
Fax: 248-852-4466
E-mail: info@oxfordbiomed.com
Made in the U.S.A.