

## Activity Assay for Total Rabbit tPa

For Research Use Only

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### INTRODUCTION

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Tissue-type Plasminogen Actvator (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.

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### PRINCIPLES OF PROCEDURE

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This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of tPa levels in biological fluid. This test kit operates on the basis of sandwich ELISA where free, latent and complexed tPa is quantified with the use of an HRP labeled secondary antibody.

The various forms of tPA present in the standard or unknown is captured by the tPA capture antibody coated on the well. 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.

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### MATERIALS PROVIDED

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Component	Volume	Storage
uPA Coated Plate		4°C
Rat tPa Activity Standard	1 vial	4°C
10x Wash Buffer	50 mL	4°C
Anti-human PAI-1 Primary Antibody	1 vial	4°C
TMB Substrate	10 mL	4°C
HRP Conjugate Secondary Antibody	1 vial	4°C

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### MATERIALS NEEDED BUT NOT PROVIDED

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- 1 N H<sub>2</sub>SO<sub>4</sub>
- TBS Buffer (see Reagent Preparation)
- Blocking Buffer (see Reagent Preparation)
- DI Water
- Microplate reader with 450 nm filter
- Microplate shaker with uniform horizontal circular movement up to 300 rpm
- Precision pipettes that range from 10 µL-1000 µL and disposable tips

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**STORAGE**

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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 unused portions of the microplate in the pouch with a desiccant at 4°C.

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**WARNINGS AND PRECAUTIONS**

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1. 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.
2. Exercise universal precautions during the performance or handling of this kit or any component contained therein.

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**PROCEDURAL NOTES**

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1. This assay should be run at room temperature.
2. Use aseptic technique when opening and dispensing reagents.
3. To minimize error due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel.

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**SAMPLE COLLECTION, STORAGE, AND PREPARATION**

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Samples should be collected using trisodium citrate, acidified citrate or Stabilyte™ (DiaPharma) collection media. Collection should be in accordance with the collection vials manufacturers instructions or in a 1:10 ratio of collection media to blood.

Immediately, upon collection of blood, the samples should be centrifuged at 3000 x g. This should ensure the removal of platelets as they can release PAI-1 that, in turn, complexes with tPA. The plasma can be transferred to a clean plastic tube and stored frozen for up to one month. Samples are stable for approximately 24 hours when stored at 4°C with the Sabilyte™ media or one month if stored at -20°C.

**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.

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**REAGENT PREPARATION**

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The following solutions should be prepared fresh before starting the assay.

1. **TBS Buffer:** 0.10 M TRIS, 0.15 M NaCl, pH 7.4
2. **Blocking Buffer:** 3% BSA in TBS Buffer.
3. **10x Wash Buffer:** Dilute to 1x prior to use. Do this by combining 1 part of 10x Wash Buffer to 9 parts of DI Water relative to the amount required for the assay, either in whole or in part.

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**ASSAY PROCEDURE**

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1. Prepare standards as indicated in the provided dilution table.  
**Note:** The standards should be applied to the plate immediately upon preparation.
2. Add 100 µL of standards or unknowns to each well omitting wells for later use. See **Scheme I** for a suggested template design.
3. Shake the plate at 300 rpm on a plate shaker for 30 minutes.
4. Wash wells according to the following wash procedure:
  - a. Remove contents of the plate by inversion into an appropriate disposal device.
  - b. Tap out remaining contents of the plate onto a lint free paper towel.
  - c. Add 300 µL of Wash Buffer to each well.

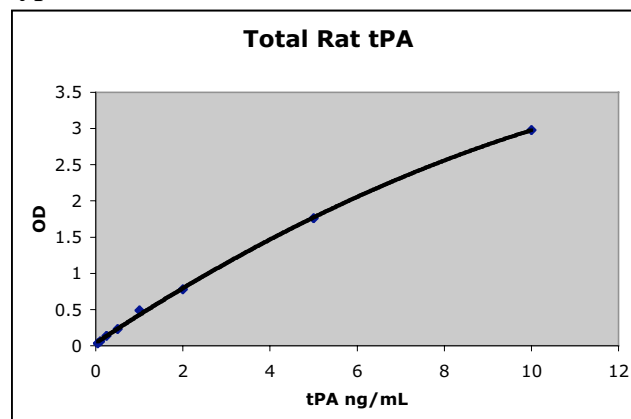
- d. Let stand for 2-3 minutes.
- e. Repeat procedure 2 more times and proceed to step “f”.
- f. Remove contents of the plate by inversion into an appropriate disposal device.
- g. Tap out the remaining contents of the plate onto a lint free paper towel and proceed to step 6.  
**Note:** The decanted wells should be void of visible moisture before proceeding. If moisture is still visible then follow step “g” until satisfactory results are obtained.
5. Make a working concentration of Primary Antibody by combining with 3% BSA Blocking Buffer. See vial label for the specific volume.
6. Add 100 µL of diluted Primary Antibody to each well.
7. Shake plate at 300 rpm on the plate shaker for 30 minutes.
8. Wash wells according to step 5 located above in this section.
9. Make a working concentration of Secondary Antibody by combining with 3% BSA Blocking Buffer. See vial label for the specific volume.
10. Add 100 µL of the working concentration Secondary Antibody Solution to each well.
11. Shake plate at 300 rpm on the plate shaker for 30 minutes.
12. Wash wells according to step 5 located above in this section.
13. Add 100 µL of TMB Substrate to each well and incubate for 10 minutes. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well).
14. Stop the reaction with 50 µL per well of 1 N H<sub>2</sub>SO<sub>4</sub> and read plate at 450 nm.

**Scheme I:**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	U1
B	S0	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	U1
C	U2	U3	U4	U5	U6	U7	U8	U9	U10	U11	U12	U13
D	U2	U3	U4	U5	U6	U7	U8	U9	U10	U11	U12	U13
E	U14	U15	U16	U17	U18	U19	U20	U21	U22	U23	U24	U25
F	U14	U15	U16	U17	U18	U19	U20	U21	U22	U23	U24	U25
G	U26	U27	U28	U29	U30	U31	U32	U33	U34	U35	U36	BLK
H	U26	U27	U28	U29	U30	U31	U32	U33	U34	U35	U36	BLK

**CALCULATIONS**

1. Subtract the average O.D. value of the blank wells (BLK) from all other pairs of wells. Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.
2. Average the O.D. values for each pair of duplicate wells.
3. Plot a standard curve using the average O.D. value for each standard value versus the concentration of standard.
4. Determine the concentration of each unknown by interpolation from the standard curve.

**Typical Standard Curve:**

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**PERFORMANCE CHARACTERISTICS**

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Assay Range: 0.0-10 ng/mL

Samples with tPa levels higher than 10 ng/mL should be diluted in similar media devoid of active tPa.

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**REFERENCES**

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