ab198510 –

Tissue Plasminogen Activator (TPA) Rat ELISA Kit

Instructions for Use

For the quantitative measurement of active Tissue Plasminogen Activator (TPA) in rat plasma and other biological fluids.

This product is for research use only and is not intended for diagnostic use.

Version 2 Last Updated 15 January 2019

Table of Contents

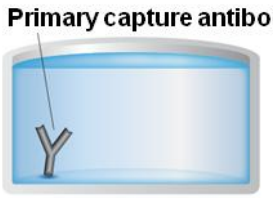
1. BACKGROUND

Abcam's Tissue Plasminogen Activator (TPA) *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of active Tissue Plasminogen Activator (TPA) in rat plasma and other biological fluids.

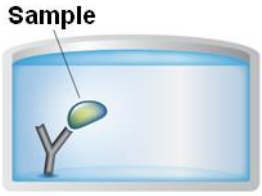
Functionally active rat TPA will form a covalent complex with the biotinylated Human PAI-1 which is bound to the avidin on the plate. Only free active TPA will react with the PAI-1 bound to the plate. After appropriate washing steps, polyclonal anti-TPA primary antibody binds to the captured TPA. Excess antibody is washed away and bound primary antibody is then reacted with the secondary antibody conjugated to horseradish peroxidase. Following an additional washing step, TMB substrate is used for color development at 450 nm. The amount of color development is directly proportional to the concentration of active TPA in the sample.

TPA is a serine protease that converts plasminogen to the active serine protease plasmin in the blood fibrinolytic system. It also plays an important role in the removal of incipient thrombi. TPA is widely used for the thrombolytic treatment of acute myocardial infarction.

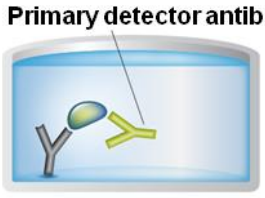
2. ASSAY SUMMARY



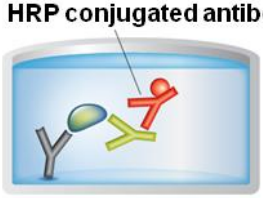
Prepare all reagents, samples, and controls as instructed. Plate is supplied pre-coated with capture antibody.



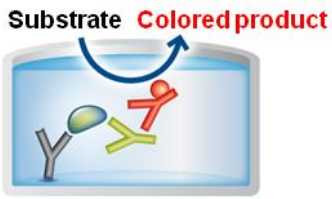
Add sample to appropriate wells. Incubate at room temperature.



Add primary detection antibody. Incubate at room temperature.



Aspirate and wash each well. Add HRP conjugated secondary antibody, which binds the primary antibody. Incubate at room temperature.



Aspirate and wash each well. Add developing solution until color develops and then add the stop solution. Immediately begin recording the color development.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store all kit components at +2-8°C upon arrival.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Reagent Preparation section.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Avidin pre-coated 96 well microplate (12 x 8 well strips)	1X 96 tests	+2-8°C
10X Wash Buffer	1X 50 mL	+2-8°C
10X TBS buffer, pH 7.4	1X 5 mL	+2-8°C
Biotinylated Human PAI-1	1 Vial	+2-8°C
Rat TPA activity standard	1 Vial	+2-8°C
Anti-TPA primary antibody	1 Vial	+2-8°C
HRP Secondary antibody	1X 10 µL	+2-8°C
TMB substrate solution	1X 10 mL	+2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microtiter plate shaker capable of 300 rpm uniform horizontally circular movement
- Manifold dispenser/aspirator or automated microplate washer
- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and Pipette tips
- Deionized or distilled water
- Polypropylene tubes for dilution of standard
- Paper towels or laboratory wipes
- 1N H_2SO_4 or 1N HCl
- Bovine Serum Albumin Fraction V (BSA)
- Tris(hydroxymethyl)aminomethane (Tris)
- Sodium Chloride (NaCl)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Always pour peroxidase substrate out of the bottle into a clean test tube. Do not pipette out of the bottle as contamination could result.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

9.1. **1X Wash Buffer**

Dilute 50 mL of 10X Wash Buffer with 450 mL of deionized water.

9.2. **1X TBS Buffer**

0.1M Tris, 0.15M NaCl, pH 7.4

9.3. **1X Blocking Buffer**

3% BSA (w/v) in TBS.

9.4. **TPA Primary Antibody**

Reconstitute TPA Primary Antibody by adding 10 mL of 1X Blocking Buffer directly to the vial and agitate gently to completely dissolve contents**.** Reconstituted TPA Primary Antibody may be stored at -80°C for later use.

9.5. **Reconstituted Human PAI-1**

Add 10 mL 1X Blocking Buffer directly to the Biotinylated Human PAI-1 vial and agitate gently to completely dissolve contents.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

10.1 Reconstitute TPA Standard by adding 1 mL of 1X Blocking Buffer directly to the vial and agitate gently to completely dissolve contents. This will result in a 1,000 ng/mL **Standard #1**.

10.2 Label eleven further tubes # 2-12.

10.3 Prepare **Standard #2**, in tube #2 by adding 50 µL (1,000 ng/mL) **Standard #1** to 950 µL 1X Blocking Buffer and mix gently and thoroughly.

10.4 Using the table below as a guide, prepare further serial dilutions using 1X Blocking Buffer as diluent.

10.5 1X Blocking Buffer serves as the zero standard, 0 ng/mL (tube #12).

Standard Dilution Preparation Table

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of 1X Blocking Buffer (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	See section 10.1				1,000
2	Standard #1	50	950	1,000	50
3	Standard #2	500	500	50	25
4	Standard #3	400	600	25	10
5	Standard #4	500	500	10	5
6	Standard #5	400	600	5	2
7	Standard #6	500	500	2	1
8	Standard #7	500	500	1	0.5
9	Standard #8	500	500	0.5	0.25
10	Standard #9	400	600	0.25	0.1
11	Standard #10	500	500	0.1	0.05
12 (Blank)	-	-	500	-	0

11. SAMPLE COLLECTION AND STORAGE

11.1 Collect plasma using EDTA or citrate as an anticoagulant. Heparinated plasma is not recommended.

11.2 Centrifuge for 15 minutes at 1,000 x g within 30 minutes of collection. It is important to ensure a platelet free preparation as platelets can release PAI-1, which in turn could potentially form a complex with TPA.

11.3 Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles. Serum and cell culture media at neutral pH may also be used.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well strips should be returned to the plate packet and stored at 2-8°C.
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Return any unused microplate strips to the plate pouch with desiccant. Reconstituted standards and primary may be stored at -80°C for later use. Do not freeze-thaw the standard and primary antibody more than once. Store all other unused kit components at 2-8°C.**

13.1 Prepare all reagents, working standards, and samples as directed in the previous sections.

13.2 Remove microtiter plate from bag and add 100 µL reconstituted Biotinylated Human PAI-1 to all wells.

13.3 Shake plate at 300 rpm for 30 minutes.

13.4 Wash wells three times with 300 µL 1X Wash Buffer. Remove excess 1X Wash Buffer by gently tapping plate on paper towel.

13.5 If using acidified citrate samples with a pH lower than 6.0, add 30 µL of 10X TBS buffer in each well and construct the standard curve in the same format. If using samples at a neutral pH, this step can be omitted.

13.6 Add 100 µL TPA Standards (in duplicate) and samples to wells. Carefully record position of Standards and Samples.

13.7 Shake plate at 300 rpm for 30 minutes.

13.8 Wash wells three times with 300 µL wash buffer. Remove excess wash by gently tapping plate on paper towel.

NOTE: The assay measures active TPA in the 0.05-50 ng/ mL range. If the unknown is thought to have high TPA levels, dilutions may be made in blocking buffer. Plasma and serum samples should be applied directly to the plate without dilution.

13.9 Add 100 µL TPA Primary Antibody to all wells.

13.10 Shake plate at 300 rpm for 30 minutes.

13.11 Wash wells three times with 300 µL 1X Wash Buffer. Remove excess 1X Wash Buffer by gently tapping plate on paper towel.

13.12 Dilute 1 µL of HRP conjugated Secondary Antibody in 10 mL of 1X Blocking Buffer and add 100 µL to all wells.

13.13 Shake plate at 300 rpm for 30 minutes.

13.14 Wash wells three times with 300 µL 1X Wash Buffer. Remove excess 1X Wash Buffer by gently tapping plate on paper towel.

13.15 Add 100 µL TMB substrate to all wells and shake plate for 2-10 minutes. Substrate will change from colorless to different strengths of blue.

13.16 Quench reaction by adding 50 µL of 1N H_2SO_4 or HCl stop solution to all wells when samples are visually in the same range as the standards. Add stop solution to wells in the same order as substrate upon which color will change from blue to yellow. Mix thoroughly by gently shaking the plate.

13.17 Set the absorbance at 450 nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450 nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A_{450}).

14. CALCULATIONS

Plot A_{450} against the amount of TPA in the standards. Fit a straight line through the linear points of the standard curve using a linear fit procedure if unknowns appear on the linear portion of the standard curve. Alternatively, create a standard curve by analyzing the data using a software program capable of generating a four parameter logistic (4PL) curve fit. The amount of TPA in the Samples can be determined from this curve. If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

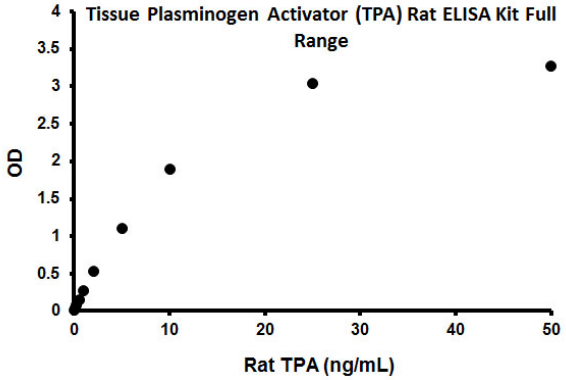
NOTE: No specific data has been reported for rat TPA concentrations. Please refer to references for mouse TPA. The concentration level of TPA antigen in mouse plasma has been reported to be 2.5±1.0 ng/mL.

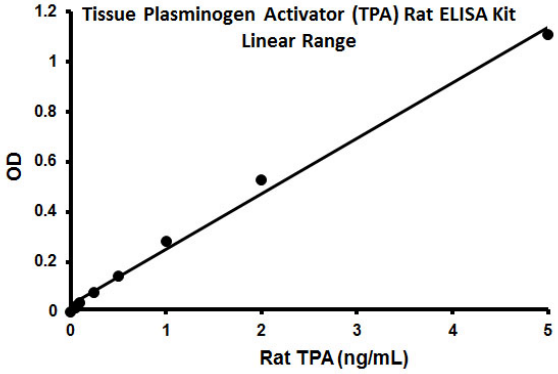
Abnormalities in TPA levels have been reported in the following conditions:

- Ischemic Diseases: TPA may affect the course of ischemic diseases.
- Pathological Infarction: TPA may prevent or limit pathological infarction and improve neurological functions. Usage of TPA at the onset of ischemic stroke improves clinical outcome.
- Blood-Brain Barrier: TPA is necessary and sufficient to directly increase the vascular permeability in the early stages of BBB opening.
- Venous Thrombosis: Locally applied TPA reduces thrombus formation after vascular injury.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Graphs provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.





16. TYPICAL SAMPLE VALUES

INTRA-ASSAY PRECISION

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Sample	1	2	3
n	20	20	20
Mean (ng/mL)	0.80	4.25	13.2
Standard Deviation	0.034	0.225	0.553
CV (%)	4.28	5.29	4.20

INTER-ASSAY PRECISION

Three samples of known concentration were tested in ten independent assays to assess inter-assay precision.

Sample	1	2	3
n	10	10	10
Mean (ng/mL)	1.37	4.80	13.3
Standard Deviation	0.06	0.34	0.75
CV (%)	4.71	7.13	5.65

SENSITIVITY

The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of twenty-two zero standard replicates (range OD_{450}: 0.059-0.071) and calculating the corresponding concentration. The MDD was 0.043 ng/mL.

RECOVERY

The recovery of antigen spiked to levels throughout the range of the assay in blocking buffer was evaluated.

Sample	1	2	3	4
n	4	4	4	4
Mean (ng/mL)	0.20	0.45	1.43	6.19
Average % Recovery	102	106	97	90
Range	98 - 105%	94 - 118%	93 - 106%	86 - 100%

LINEARITY OF DILUTION

To assess the linearity of the assay, pooled citrated rat plasma samples containing high concentrations of antigen were serially diluted to produce samples with values within the dynamic range of the assay. Plasma samples did not demonstrate linearity and should be analyzed without dilution.

17. ASSAY SPECIFICITY

This assay recognizes natural and recombinant active rat TPA. Pooled normal plasma from mouse was assayed and significant cross-reactivity was observed. Pooled normal plasma from pig was assayed and minor cross-reactivity was observed. Pooled normal plasma from Human, horse, dog, rabbit and sheep was assayed and no significant cross-reactivity was observed.

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting Improper standards dilution	Check pipettes Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components 2-8°C. Keep substrate solution protected from light.

19. NOTES

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