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Molecular® Innovations

Human tPA Activity ELISA KIT

Catalog # HTPAKT

Strip well format. Reagents for up to 96 tests.

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INTENDED USE

This human tissue-type plasminogen activator (tPA) activity assay is intended for the quantitative determination of active tPA in human plasma and other biological fluids. **For research use only.**

BACKGROUND

tPA is a serine protease that catalyzes the activation of plasminogen to plasmin [1]. 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].

ASSAY PRINCIPLE

Functionally active tPA will form a covalent complex with biotinylated human PAI-1 which is bound to the avidin coated on the microtiter plate. Complexed tPA will not bind to the PAI-1 and will not be detected by the assay. After appropriate washing steps, polyclonal anti-human tPA primary antibody binds to the captured tPA. Excess antibody is washed away and bound polyclonal antibody is reacted with the secondary antibody conjugated to horseradish peroxidase. Following an additional washing step, TMB is used for color development at 450nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of human tPA. The amount of color development is directly proportional to the concentration of active tPA in the sample.

STANDARD CALIBRATION

The tPA activity standard provided contains single chain human tPA and is calibrated against the International Standard for tPA distributed by NIBSC (98/714), South Mimms, Potters Bar, Hertfordshire, UK.

Lot 413L-A: 1 IU = 1.64 ng

REAGENTS PROVIDED

- **96-well avidin coated microtiter strip plate** (removable wells 8x12) containing avidin, blocked and dried.
- **10X Wash Buffer:** 1 bottle of 50ml
- **General Assay Diluent:** 1 bottle of 10ml
- **Biotinylated Human PAI-1:** 1 vial lyophilized protein
- **Human tPA activity standard:** 1 vial lyophilized standard
- **Anti-human tPA primary antibody:** 1 vial lyophilized polyclonal antibody
- **Anti-rabbit horseradish peroxidase-conjugated secondary antibody:** 1 vial concentrated HRP labeled antibody
- **TMB substrate solution:** 1 bottle of 10ml solution

STORAGE AND STABILITY

Store all kit components at 4°C upon arrival. Return any unused microplate strips to the plate pouch with desiccant. Reconstituted PAI-1, standard and primary may be stored at -80°C for later use. Do not freeze-thaw the PAI-1, standard and primary antibody more than once. Store all other unused kit components at 4°C. This kit should not be used beyond the expiration date.

OTHER REAGENTS AND SUPPLIES REQUIRED

- 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)

PRECAUTIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
- Always pour peroxidase substrate out of the bottle into a clean test tube. Do not pipette out of the bottle as contamination could result.
- Keep plate covered except when adding reagents, washing, or reading.
- DO NOT pipette reagents by mouth and avoid contact of reagents and specimens with skin.
- DO NOT smoke, drink, or eat in areas where specimens or reagents are being handled.

PREPARATION OF REAGENTS

- **TBS buffer:** 0.1M Tris, 0.15M NaCl, pH 7.4
- **Blocking buffer (BB):** 3% BSA (w/v) in TBS
- **1X Wash buffer:** Dilute 50ml of 10X wash buffer concentrate with 450ml of deionized water

SAMPLE COLLECTION

For best results collect 9 volumes of blood in 1 volume of 0.1M acidified citrate [5]. The low pH of the resulting plasma insures that plasma PAI-1 is inhibited from quenching tPA activity [6]. Immediately after collection of blood, samples must be centrifuged at 2500Xg for 15 minutes. It is important to ensure a platelet free preparation as platelets can release PAI-1 which could potentially form a complex with active tPA. The plasma must be transferred to a clean plastic tube and stored on ice prior to analysis. The tPA activity samples collected 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. Samples of human plasma in citrate or EDTA may be assayed with this kit. Plasma in heparin is not recommended. Serum and cell culture media at neutral pH may also be used.

ASSAY PROCEDURE

Perform assay at room temperature. Vigorously shake plate (300rpm) at each step of the assay.

Biotinylated Human PAI-1 Addition

Add 10ml blocking buffer directly to the biotinylated human PAI-1 vial and agitate gently to completely dissolve contents. Remove microtiter plate from bag and add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Preparation of Standard

Reconstitute standard by adding 10ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. This will result in a 61 IU/ml standard solution. Dilute 100µl of 61 IU/ml standard into 900µl of blocking buffer to generate a 6.1 IU/ml standard.

Dilution table for preparation of human tPA standard:

tPA concentration (IU/ml)	µl of 6.1 IU/mL tPA standard	µl of blocking buffer	Total volume (µl)
1	100	510	610
0.5	50	560	610
0.4	40	570	610
0.25	25	585	610
0.1	10	600	610
0.05	5	605	610
0.02	2	608	610
0.01	1	609	610
0	0	500	500

NOTE: DILUTIONS FOR THE STANDARD CURVE AND ZERO STANDARD MUST BE MADE AND APPLIED TO THE PLATE IMMEDIATELY.

Standard and Unknown Addition

If samples are at neutral pH, add 100µl of tPA standards (in duplicate) and unknowns to wells. If the pH of samples is below pH 6.0, first add 40µl of General Assay Diluent to all wells then add 60µl of tPA standards (in duplicate) and unknowns to wells. Carefully record position of standards and unknowns. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: The assay measures active tPA in the 0.01-1 IU/ml range. If the unknown is thought to have high tPA levels, dilutions may be made in blocking buffer.

Primary Antibody Addition

Reconstitute primary antibody by adding 10ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. Add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Secondary Antibody Addition

Dilute 2µl of conjugated secondary antibody in 10ml of blocking buffer and add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Substrate Incubation

Add 100µl TMB substrate to all wells and shake plate for 5-15 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50µl 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.

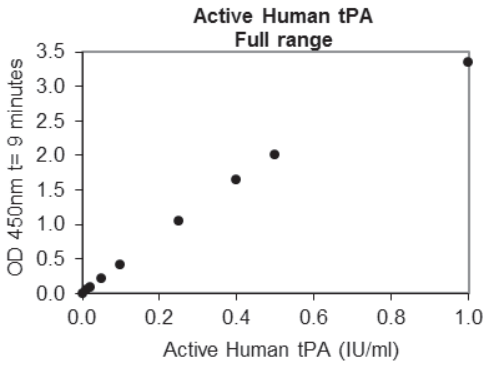
Measurement

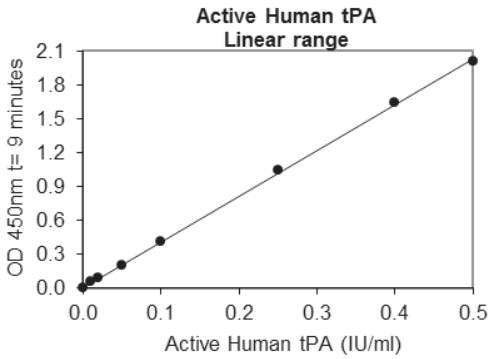
Set the absorbance at 450nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A_{450}).

Calculation of Results

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 unknowns can be determined from this curve. If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

A typical standard curve (EXAMPLE ONLY):





EXPECTED VALUES

The basal level of tPA in healthy humans has been reported to be between 0.2-2 IU/mL [8]. In house testing of pooled normal human plasma found concentrations of 0.02 IU/mL.

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

Sensitivity: 0.006 IU/ml.

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

Sample	High	Medium	Low
n	20	20	20
CV (%)	3.8	4.0	9.8

Inter-assay Precision: These studies are currently in progress. Please contact us for more information.

Recovery: These studies are currently in progress. Please contact us for more information.

Linearity: These studies are currently in progress. Please contact us for more information.

Specificity: These studies are currently in progress. Please contact us for more information.

DISCLAIMER

This information is believed to be correct but does not claim to be all-inclusive and shall be used only as a guide. The supplier of this kit shall not be held liable for any damage resulting from handling of or contact with the above product.

REFERENCES

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Example of ELISA Plate Layout
96 Well Plate: 18 Standard wells, 78 Sample wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.01 IU/ml	0.02 IU/ml	0.05 IU/ml	0.1 IU/ml	0.25 IU/ml	0.4 IU/ml	0.5 IU/ml	1 IU/ml			
B	0	0.01 IU/ml	0.02 IU/ml	0.05 IU/ml	0.1 IU/ml	0.25 IU/ml	0.4 IU/ml	0.5 IU/ml	1 IU/ml			
C												
D												
E												
F												
G												
H												