*****REPRESENTATIVE DATASHEET*****

Matched-Pair Antibody Set for ELISA of human

tissue Plasminogen Activator antigen (tPA)

Sufficient reagent for 5 x 96 well plates

Product #:	TPA-EIA
Lot #:	SAMPLE
Expiry Date:	SAMPLE

Store at –10 to -20°C

For Research Use Only Not for use in diagnostic procedures.

Description of tissue Plasminogen Activator (tPA)

Tissue-type plasminogen activator (tPA) is one of two major physiologic activators of plasminogen in plasma. It is a serine protease of 68 kDa produced primarily in endothelial cells but is also present in monocytes and megakaryocytes. Normal plasma tPA antigen concentrations have been reported from 20 ng/ml to 5 µg/ml, depending on the assay used, but typically most of the tPA (> 90%) is in complex with it's primary inhibitor, plasminogen activator inhibitor-1 (PAI-1). Structurally, tPA is a single-chain enzyme that consists of a catalytic domain followed by two kringle structures, an EGF domain and a finger domain. The activation of plasminogen by tPA is dependent on the presence of a fibrin cofactor. The binding of both tPA and plasminogen to fibrin is mediated in part through lysine binding sites within the kringle structures of both enzyme and substrate, but also through the finger domain of tPA. Activation of plasminogen by tPA occurs by cleavage after residue Arg^{560} to produce the two-chain active serine protease plasmin. The activity of tPA is regulated in part by a very short half life in circulation (t½ of ~4 minutes) and by circulating protease inhibitors PAI-1 and to a lesser extent $\alpha_2$macroglobulin[1-3].

Principle of Sandwich-style ELISA

Affinity-purified antibody to tPA is coated onto the wells of a microtitre plate. Any remaining binding sites on the plastic wells are blocked with an excess of bovine serum albumin. The plates are washed and plasma or other fluids containing tPA are applied. The coated antibody will capture the tPA in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to tPA is added to the plate to bind to the captured tPA. After washing the plate to remove unbound conjugated antibody, the peroxidase activity is expressed by incubation with o-phenylenediamine (OPD). After a fixed development time the reaction is quenched with the addition of H_2SO_4 and the colour produced is quantified using a microplate reader. The colour generated is proportional to the concentration of tPA present in the sample.

COA CHROM DIAGNOSTICA

Supplied Materials:

1. Capture Antibody (TPA-EIA-C): One yellow-capped vial containing 0.5 ml of polyclonal affinity purified anti-tPA antibody for coating plates.
2. Detecting Antibody (TPA-EIA-D): One red-capped vial containing 0.5 ml of peroxidase conjugated polyclonal antitPA antibody for detection of captured tPA.

Note: Antibodies are supplied in a 50% (v/v) glycerol solution for storage at –10 to -20°C. Keep vials tightly capped. Do not store in frost-free freezers.

Materials Required but not Provided:

1. Coating Buffer: 50 mM Carbonate 1.59g of Na_2CO_3 and 2.93g of $NaHCO_3$ up to 1 litre. Adjust pH to 9.6. Store at 2-8°C up to 1 month.

2. **PBS:** (base for wash buffer and blocking buffer) 8.0g NaCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 and 0.2g KCl, up to 1 litre. Adjust pH to 7.4, if necessary. Store up to 1 month at 2-8°C, discard if there is evidence of microbial growth.

3. **Wash Buffer:** PBS-Tween (0.1%,v/v) To 1 litre of PBS add 1.0 ml of Tween-20. Check that the pH is 7.4. Store at 2-8°C up to 1 week.

4. **Blocking Buffer:** PBS-BSA (1%, w/v) Dissolve 2.5 g of Bovine Serum Albumin (Sigma-RIA grade) in 200 ml of PBS. Adjust pH to 7.4, if required, then make up to 250 ml with PBS. Aliquot and store frozen at -20°C.

5. **Sample Diluent:** HBS-BSA-T20 5.95g HEPES (free acid), 1.46 g NaCl, 2.5 g Bovine Serum Albumin (Sigma, RIA grade) dissolved in 200 ml H_2O. Add 0.25 ml of Tween-20, check and adjust pH to 7.2 with NaOH, then make up to a final volume of 250 ml with H_2O. Aliquot and store frozen at -20°C.

6. **Substrate Buffer:** Citrate-Phosphate buffer pH 5.0 2.6g Citric acid and 6.9g Na_2HPO_4 up to a final volume of 500 ml with purified H_2O. Store at 2-8°C up to 1 month.

7. **OPD Substrate:** (o-Phenylenediamine.2HCl) TOXIC! (5mg tablets: Sigma # P-6912). Make up immediately before use. Dissolve 5mg OPD in 12 ml substrate buffer then add 12 µl 30% H_2O_2. Do not store.

8. **Stopping Solution:** 2.5 M H_2SO_4 CAUTION: VERY CORROSIVE! GENERATES HEAT ON DILUTION! Where stock sulphuric acid is 18 Molar, add 13.9 ml to 86 ml H_2O. Store at room temperature.

9. **Other:** Microplates, 96-well Falcon PVC (Product # 35-3912) tPA reference standard (American Diagnostica, cat # 178) tPA/PAI-1 deficient plasma (Trinity Biotech, cat # 101-442) Microplate washer (optional) Microplate reader.

Assay Procedure:

1. Coating of plates: Dilute the capture antibody 1/100 in coating buffer (in a polypropylene tube) and immediately add 100 µl per well in the plate. Incubate for 2 hours at ambient temperature or overnight at 2-8$_o$C.
2. Blocking: Empty contents of plate and add 150 µl of blocking buffer to every well and incubate for 60 minutes @ 22$_o$C. Wash plate X 3 with wash buffer.
3. Preparation of tPA Reference Standards: Reconstitute vials of tPA standard and tPA/PAI-1 deficient plasma according to manufacturers instructions. After reconstitution, dilute the tPA standard into tPA/PAI-1 deficient plasma to achieve six reference standard plasmas with final tPA concentrations of 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/ml respectively.
4. Samples: Reference plasmas prepared in step 3 and test plasmas are diluted 1/4 and 1/8 in HBS-BSA-T20 sample diluent. Samples should be run in duplicate. Apply 100 µl/well and incubate plate @ 22$_o$C for 90 minutes. Wash plate X 3 with wash buffer.
5. Detecting Antibody: Dilute the detecting antibody 1/100 in HBS-BSA-T20 sample diluent and apply 100 µl to each well. Incubate plate @ 22$_o$C for 90 minutes. Wash plate X 3 with wash buffer.
6. OPD Substrate: Apply 100 µl of freshly prepared OPD substrate to every well. Allow colour to develop for 5-10 minutes then stop colour reaction with the addition of 50 µl/well of 2.5 M H_2SO_4. The plate can be read at a wavelength of 490 nm.

Calculation of Results:

The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit [4,5]. In general, the simplest model that defines the concentration-response relationship should be used [6]. The "back-fit" test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits[6]. The coefficient of determination (R^2) is a valuable indicator of the overall fit, but should not be used by itself in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone [5,6]

In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed using serial dilutions of normal pooled plasma, will produce a correlation coefficient (R^2) of at least 0.980 using a log-log fit, and an R^2 of at least 0.990 using a 4-parameter logistic curve fit algorithm. However, the performance characteristics of inhouse assays developed using this product in other laboratories may vary slightly from ours. Different curve fitting methods may be employed but we recommend that the back-fit test be applied as evidence that the fitting method is appropriate.

Technical Notes:

-This paired antibody product is intended to facilitate the end user in establishing an in-house immunoassay for research purposes only. It must not be used for diagnostic applications. Assay validation is the responsibility of the end user and should be done according to user-defined protocols[6].

-Reference calibrators should be of the same matrix and anticoagulant as the samples to be tested (example serum or plasma, citrate or EDTA).
-Do not use samples diluted less than 1/2, as falsely high readings may result.
-The optimal colour development time should be determined empirically as the time required to obtain an absorbance of at least 1.000 at 490 nm for the 100% reference point, not to exceed 20 minutes.
-Rheumatoid factor in samples may interfere in ELISA by binding to the capture and/or detecting antibodies.
-The wells should not be allowed to become dry. Keep plate covered or in a humid chamber during incubations.
-Antibodies are supplied in a 50% glycerol solution and can be centrifuged briefly in a micro-centrifuge to gather residual reagent from the cap and walls of the tube.

References:

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3. 3 Giles AR, Nesheim, *et al*; The fibrinolytic Potential of the Normal Primate following the Generation of Thrombin *In Vivo*; Thrombosis and Haemostasis 63, pp. 476-481, 1990.
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5. NCCLS. Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guidline – Second Edition. NCCLS Document EP6-P2 (ISBN 1 56238-446-5, NCCLS, Wayne, Pennsylvania USA, 2001.
6. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available on the internet: www.fda.gov/cder/guidance/index.htm