REPRESENTATIVE DATASHEET

Matched-Pair Antibody Set for ELISA of human
Thrombin-Heparin Cofactor II complex (THCII)

Sufficient reagent for 5 x 96 well plates

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

Store at -10 to -20°C

For Research Use Only Not for use in diagnostic procedures.

Description of Thrombin-Heparin Cofactor II Complex

The activation of coagulation ultimately leads to the activation of prothrombin to the enzyme thrombin. Unless regulated, thrombin will act on its natural substrates that include fibrinogen, factor V, factor VIII, factor XIII, Protein C, TAFI as well as specific receptors on platelets and endothelial cells. The activity of thrombin in plasma is regulated in part through interaction with protease inhibitors. Based on kinetic rates and physiological concentrations, the primary inhibitor of thrombin in plasma is antithrombin (ATIII), followed by heparin cofactor II (HCII) and $\alpha_2$macroglobulin.

The thrombin-heparin cofactor II complex (T-HCII) results when thrombin cleaves a scissile bond near the C-terminus of HCII, forming a covalent, 1:1 acyl enzyme intermediate with HCII with an apparent mass of 102 kDa. Calcium is not required for this interaction, but the rate of thrombin inhibition by HCII can be accelerated 1000-fold by optimal concentrations of heparin. Unlike Antithrombin, thrombin inhibition by HCII is also enhanced by dermatan sulphate. T-HCII complexes are cleared from circulation by serpin-enzyme complex receptors on the surface of hepatocytes, with a half-life of 10 minutes[1-3].

Principle of Sandwich-style ELISA

Affinity-purified antibody to human thrombin is coated onto the wells of a microtitre plate. Any remaining binding sites on the plastic wells are blocked with bovine serum albumin. The plates are washed and plasma or other fluids are applied. The coated antibody will capture the thrombin and thrombin-inhibitor complexes in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to HCII is added to the plate to bind to the captured THCII complexes. 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 THCII complex present in the sample.

Supplied Materials:

1. Capture Antibody (THCII-EIA-C): One yellow-capped vial containing 0.5 ml of polyclonal affinity purified anti-thrombin antibody for coating plates.

2. Detecting Antibody (THCII-EIA-D): One red-capped tube containing 0.5 ml of peroxidase conjugated polyclonal anti-HCII antibody for detection of captured THCII complex.

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 (2%, w/v) Dissolve 5.0 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. **Materials for making TAT reference standards:**
- Purified human Heparin Cofactor II (Affinity, cat# HHCII)
- HCII deficient plasma, lyophilized (Affinity, cat# HCII-LDP).
- Human thrombin available from Enzyme Research Labs, South Bend, IN (tel: 574-288-2268).

10. **Other:** Microplates, 96-well Immulon 4-HBX (http://www.labsystems.fi) Microplate washer (optional) Microplate reader

Assay Procedure:

1. Preparation of Thrombin-HCII complex reference standard: Purified HCII (330 µg/mL = 5 µM) in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 1 mM EDTA and 0.05 U/mL heparin, is incubated with a limiting amount of thrombin (37 µg/mL = 1 µM) at 37$_o$C for 30 minutes. Complete inhibition should be confirmed by plasma clot time or chromogenic assay. If all thrombin is completely inhibited, the concentration of T-HCII complex is 1 µM. A series of standards can be made by diluting this stock T-HCII complex into HCII-deficient plasma. Prepare standards containing 600, 200, 60 and 20 pM respectively.

2. **Coating of plates:** Dilute the capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 µl to every well in the plate. Incubate 2 hours at room temperature or overnight at 2-8$_o$C.

3. **Blocking:** Empty contents of plate and add 150 µl of blocking buffer to every well and incubate for 90 minutes @ 22$_o$C. Wash plate X 3 with wash buffer.

4. Standards and Test **Samples:** Test samples and the reference standards prepared above are each diluted 1/4 in sample diluent. Apply 100 µl/well and incubate plate @ 22$_o$C for 120 minutes. Wash plate X 3 with wash buffer.

5. **Detecting Antibody:** Dilute the detecting antibody 1/100 in sample diluent and apply 100 µl to each well. Incubate plate @ 22$_o$C for 60 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 10-15 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 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 5parameter 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 constructed as described above 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 4parameter logistic curve fit algorithm. However, the performance characteristics of in-house 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 standards may not be stable to freeze-thaw. If standards are aliquoted and stored frozen, the concentration of complex should be retested against freshly prepared standards.

-Any test plasma that assays above the readable range should be diluted into HCII-deficient plasma and retested.

-Do not use samples diluted less than 1/4, 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.

-Stock 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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4. Nix,B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.
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