Enzyme Research Laboratories
Global Coag

Matched Pair Antibody Set for ELISA of Human Prothrombin antigen (FII)

Sufficient reagent for 5 x 96 well plates

Catalogue: FII-EIA
Lot: SAMPLE
Expiry date: **/****
Store at -10 to -20°C.

For Research Use Only ~ Not for use in diagnostic procedures.

Description of Prothrombin (F.II)

Prothrombin (factor II, F.II) is a vitamin K dependant glycoprotein produced in the liver. The concentration of prothrombin in plasma is ~100 ug/ml (~1.4 uM). Prothrombin is a single chain molecule with a molecular weight of 72 kDa. Prothrombin consists of a catalytic domain followed by two kringle structures and an amino-terminal domain containing 10 y-carboxy-glutamic acid (gla) residues. The gla residues allow Prothrombin to bind to membranes that contain acidic phospholipids in a calcium dependant manner. The binding to membranes is required for effective presentation of Prothrombin as a substrate for activation by the prothrombinase complex, which consists of activated factor X, activated cofactor V and calcium on phospholipid membrane. Activation by prothrombinase occurs by sequential cleavage after residue Arg^{320} then after Arg^{271} to produce the active protease α-thrombin (37 kDa) and the by-product prothrombin fragment 1.2 (35 kDa). The product thrombin further cleaves prothrombin fragment 1.2 after residue Arg155 into individual prothrombin fragments 1 and 2. The activity of α-thrombin in plasma is inhibited primarily by antithrombin and the rate of inhibition is accelerated 1000-fold in the presence of optimal concentrations of heparin. Other physiological inhibitors of thrombin in the absence of heparin include $\alpha_2$macroglobulin and $\alpha_1$antitrypsin [1-3].

Principle of Sandwich-style ELISA

Affinity-purified antibody to F.II is coated onto the wells of a microtitre plate. The plates are washed and plasma or other fluids containing F.II are applied. The coated antibody will capture the F.II in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to F.II is added to the plate to bind to the captured F.II. 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 F.II present in the sample.

Supplied Materials:

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

2. **Detecting Antibody (FII-EIA-D):** One red-capped vial containing 0.5 ml peroxidase conjugated polyclonal anti-FII antibody for detection of captured FII.

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

Materials Required but not Provided:

1. **Coating Buffer:** (50 mM Carbonate)
1.59 g of Na_2CO_3 and 2.93 g 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)
8.0g NaCl and 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 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 pH is 7.4. Store at 2-8°C up to 1 week.

4. **Sample Diluent:** HBS-BSA-T20
5.95 g 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.

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

6. **OPD Substrate:** (O-Phenylenediamine) Toxic!
Available in 5 mg tablets from Sigma # P-6912: Make up immediately before use, do not store. Dissolve 5 mg OPD in 12 ml substrate buffer then add 12 ul 30 % H_2O_2.

7. **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.

8. **Other:**
Microplates, 96-well Immulon 4-HBX (http://www.labsystems.fi)
Microplate washer (optional)
Microplate reader.
Optional: VisuLize™ Buffer Pak
Optional: VisuCal™ Antigen Calibrator

Assay procedure:

1. Coating of Plates:
Dilute the capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 ul to every well in the plate. Incubate 2 hours @ 22°C.

2. Blocking:
Blocking is not required under the conditions described. Washing the plate with PBS-Tween is sufficient to block non-specific interactions. Wash the plate X 3 with wash buffer.

3. Samples:
Reference plasma is diluted 1/5,000 (100%) then serial 1/2's down to 1/160,000 (3.13%). Sample plasmas are diluted 1/10,000, 1/20,000 & 1/40,000. All dilutions are made in HBS-BSA-T20 sample diluent. Apply 100 ul/well and incubate plate at 22°C for 60 minutes. Wash X3 with wash buffer.

4. Detecting Antibody:
Dilute the detecting antibody 1/100 in HBS-BSA-T20 sample diluent and apply 100 ul to every well. Incubate plate @ 22°C for 60 minutes. Wash X3 with wash buffer.

5. OPD Substrate:
Apply 100 ul 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 ul/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 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 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 us 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 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 be 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 1/50, 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 last 1.000 at 490 nm for the 100% reference point, not to exceed 20 minutes.
› Rheumatoid factor in plasma samples can sometimes interfere in sandwich type ELISAs 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:

1. Mann KG; Prothrombin and Thrombin; in Hemostasis and Thrombosis, 3rd Edition, eds. RW Colman, J Hirsh, VJ Marder and EW Salzman, pp. 184-199, J.B. Lippincott Co., Philadelphia PA, USA, 1994.
2. Mann KG; Prothrombin; Methods in Enzymology 45, pp 123-156, 1976.
3. Downing MW, Bloom JW, Mann KG; Comparison of the Inhibition of Thrombin by Three Plasma Protease Inhibitors; Biochemistry 17, pp 2649-2653, 1978.
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 Guideline – 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

Related Products:

SAFII-IG	Sheep Anti-Human F.II, IgG from antiserum
SAFII-AP	Sheep Anti-Human F.II, affinity-purified IgG
SAFII-HRP	Sheep Anti-Human F.II, IgG-peroxidase conj
SAFII-F1AP	Sheep Anti-Human F.II Frag 1, affinity pur IgG
SAFII-F2AP	Sheep Anti-Human F.II Frag 2, affinity pur IgG
FII-EIA	Paired antibody set for ELISA of F.II, 5 x 96 well
FII-DP	Human plasma deficient in F.II, immune depleted

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