

****REPRESENTATIVE DATA SHEET****

Matched-Pair Antibody Set for ELISA of human Apolipoprotein-H (β_2 Glycoprotein I)

Sufficient reagent for 5 x 96 well plates

Product #: APOH-EIA

Lot #: **SAMPLE**

Expiry Date: **SAMPLE**

Store at -10 to -20°C

For Research Use Only Not for use in
diagnostic procedures.

Description of Apolipoprotein-H (β_2 GPI)

Apolipoprotein-H, also known as β_2 -Glycoprotein I (β_2 GPI), is a plasma glycoprotein that circulates at a concentration of 200 $\mu\text{g/ml}$ (4 μM). Synthesized in the liver, β_2 GPI is a single chain molecule of 48 kDa, consisting of five repeating internally disulphide-bonded structures referred to as sushi domains. Relative to other glycoproteins, β_2 GPI has an unusually high content of cysteine (6.2%), proline (8.3%) and carbohydrate (19%). Almost half the circulating β_2 GPI in plasma is associated with lipoproteins of all major fractions. β_2 GPI has been demonstrated to bind negatively charged phospholipids, heparin and platelets. Although the precise function(s) are as yet unknown, β_2 GPI has been demonstrated to interfere with blood coagulation by competitively binding to negatively charged phospholipid surfaces exposed during cell activation or damage. Recent evidence also implicates β_2 GPI as a cofactor recognized by anti-phospholipid antibodies present in some autoimmune disorders such as systemic lupus erythematosus (SLE)^{1,3}.

Principle of Sandwich-style ELISA

Affinity-purified antibody to β_2 GPI is coated onto the wells of a microtitre plate. The plates are washed and plasma or other fluids containing β_2 GPI are applied. The coated antibody will capture the β_2 GPI in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to β_2 GPI is added to the plate to bind to the captured β_2 GPI. 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 β_2 GPI present in the sample.

Supplied Materials:

1. Capture Antibody (APOH-EIA-C): One yellow-capped vial containing 0.5 ml of polyclonal affinity-purified anti- β_2 GPI antibody for coating plates.
2. Detecting Antibody (APOH-EIA-D): One red-capped vial containing 0.5 ml of peroxidase-conjugated polyclonal anti- β_2 GPI antibody for detection of captured β_2 GPI.

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 $2-8^\circ\text{C}$ up to 1 month.
2. **PBS:** (base for wash 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 $2-8^\circ\text{C}$, 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 $2-8^\circ\text{C}$ up to 1 week.
4. **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 .
5. **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 $2-8^\circ\text{C}$ up to 1 month.
6. **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.
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.

Assay Procedure:

- 1. 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 hrs @ 22°C or overnight at +2-8°C .
- 2. Blocking:** Blocking is not required under the conditions described. Washing the plate with PBS-Tween is sufficient to block nonspecific interactions. Wash plate X 3 with wash buffer.
- 3. Samples:** Reference plasma is diluted 1/2000 (100%) then serial 1/2's down to 1/64000 (3.13%). Sample plasmas are diluted 1/4000, 1/8000 & 1/16000. All dilutions are made in HBS-BSA-T20 sample diluent. Apply 100 µl/well and incubate plate @ 22°C for 60 minutes. Wash plate X 3 with wash buffer.
- 4. Detecting Antibody:** Dilute the detecting antibody 1/100 in HBS-BSA-T20 sample diluent and apply 100 µl to each well. Incubate plate @ 22°C for 60 minutes. Wash plate X 3 with wash buffer.
- 5. 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 H₂SO₄. 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 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

limits⁶. 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 protocols⁶.

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 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:

1. Lee, NS, Brewer HB Jr., Osborne JC Jr.; β_2 Glycoprotein I: Molecular Properties of an Unusual Apolipoprotein, Apolipoprotein H; JBC 258, pp 4765-4770, 1983.
2. Schousboe I; β_2 Glycoprotein I: A Plasma Inhibitor of the Contact Activation of the Intrinsic Blood Coagulation Pathway. Blood 66, pp 1086-1091, 1985.
3. Nimpf J, Bevers EM, Bomans PHH, Till U, Wurm H, Kostner GM, Zwaal RFA; Prothrombinase activity of human platelets is inhibited by β_2 Glycoprotein I; Biochimica et Biophysica Acta 884, pp 142-149, 1986.
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 156238-446-5, 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