Prothrombin (Factor II) IgG ELISA

Quantitative assay for anti-prothrombin IgG antibodies

For Research Use Only. Not For Use In Diagnostic Procedures.

Updates from previous version: 09/03 are non-critical to the assay procedure.

Catalog Number: 27-GD76
Size: 96 Wells
Version: 090304 - ALPCO 09/07/04

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1. Intended use

The anti-prothrombin IgG kit is a rapid ELISA method for the detection of circulating IgG antibodies to prothrombin (factor II). The components of the kit are for research use only.

2. Explanation of the Test

Most anti-phospholipid antibodies (aPL) associated with the antiphospholipid syndrome (APS) are autoantibodies with specificity towards beta-2 glycoprotein I and / or prothrombin (factor II). It is now widely accepted that autoimmune aPL specifically reacting with beta-2 glycoprotein I are mainly responsible for anti-cardiolipin reactivity and some lupus anti-coagulant activities, whereas those with specificity for prothrombin display lupus anticoagulant activity.

Significantly elevated levels of anti-prothrombin IgG have been detected in 63% of patients with APS, 33% patients with SLE, 45% patients with rheumatoid factor and in 62% of patients with positive lupus anticoagulant.

Elevated levels of anti-prothrombin antibodies, but not of anti-beta-2 glycoprotein antibodies, imply a risk of venous thrombosis or arterial thrombosis in subjects with no previous thrombosis and no anti-phospholipid antibodies. In addition, high levels of anti-prothrombin antibodies but not anti-beta-2 glycoprotein antibodies are associated with an increased risk of myocardial infarction or cardiac death.

3. Principle of the test

Diluted serum samples are incubated with prothrombin immobilised on microtitre wells. After washing away unbound serum components, rabbit anti-human IgG conjugated to horseradish peroxidase is added to the wells and this binds to surface-bound antibodies in the second incubation. Unbound conjugate is removed by washing, and a solution containing 3,3’,5,5’-tetramethylbenzidine (TMB) and enzyme substrate is added to trace specific antibody binding. Addition of Stop Solution terminates the reaction and provides the appropriate pH for colour development. The optical densities of the standards, controls and samples are measured using a microplate reader at 450nm. Optical density is directly proportional to antibody activity in the sample.

4. Materials included in the kit

- **Microplate**: 96 wells in 12 X 8 break-apart strips, pre-coated with prothrombin, with holder in a foil bag with desiccant
- **Reagent 1: Sample Diluent** 10mM Tris-buffered saline, pH 7.2 with antimicrobial agent, 50 ml, (blue), ready to use
- **Reagent 2: Wash Buffer** 150mM Tris-buffered saline with detergent, pH 7.2, 75 ml, concentrate (x15)
- **Reagent 3: Conjugate** rabbit anti-human IgG conjugated to horseradish peroxidase in protein stabilising solution and antimicrobial agent, 12 ml, (red), ready to use
- **Reagent 4: TMB Substrate** aqueous solution of TMB and hydrogen peroxide, 12 ml, ready to use
- **Reagent 5: Stop Solution** 0.25M sulphuric acid, 12 ml, ready to use
- **Standards**: 0, 10, 20, 50, 100 & 200 U/ml, 1ml of 10mM Tris-buffered saline containing human serum IgG antibodies to prothrombin, ready to use
- **Positive Control**: 1ml of 10mM Tris-buffered saline containing human serum antibodies to prothrombin ready to use
- **Negative Control**: 1ml of 10mM Tris-buffered saline containing normal human serum, ready to use
- **Instructions for use**

5. Other equipment required

1. Test tubes for dilution • graduated cylinder for preparing wash buffer • precision pipettes and disposable tips to deliver 10µl, 100µl, 1ml • EIA microplate washer or multi-channel pipette or wash bottle • distilled or de-ionised water • absorbent paper • EIA microplate reader with 450nm and optional 620nm reference filter. Alternatively, a suitable automated system may be used.
2. Instrumentation, whether manual or automated, should meet the following criteria: pipettes with better than 3% imprecision with no carry over between pipetting steps; microplate washers should remove 99% of fluid; automated machines should minimise time between washing and adding the next reagent.

This protocol is for reference purposes only. DO NOT use this copy to run your assay; use the protocol included with the kit ONLY.
6. Precautions

6.1 Safety Precautions

1. All reagents in this kit are for research use only.
2. Only experienced laboratory personnel should use this test. The test protocol must be followed strictly.
3. All human source material used in the preparation of standards and controls for this product have been
tested and found negative for antibodies to HIV, HbsAg and HCV. No test method, however, can offer
complete assurance that infectious agents are absent. Therefore, all reagents containing human
material should be handled as if potentially infectious. Operators should wear gloves and protective
clothing when handling any patient sera or serum based products.
4. Reagents of this kit contain antimicrobial agents and the TMB Substrate solution contains 3,3',5,5'-
tetramethylbenzidine. Avoid contact with the skin and eyes. Rinse immediately with plenty of water if
any contact occurs.
5. The Stop Solution contains 0.25M sulphuric acid. Avoid contact with skin and eyes. Rinse
immediately with plenty of water if contact occurs.
6. Any liquid that has been brought into contact with potentially infectious material has to be discarded in
a container with a disinfectant. Disposal must be performed in accordance with local legislation.

6.2 Technical Precautions

1. Strips and solutions should not be used if the foil bag is damaged or liquids have leaked.
2. Allow all reagents and the microplate to reach room temperature before use. Ensure that the
microplate foil bag containing any unused strips is well sealed and contains the desiccant to avoid
moisture. Store at 2 – 8°C after use.
3. Include the Positive and Negative Control in every test run to monitor for reagent stability and correct
assay performance.
4. Strictly observe the indicated incubation times and temperature.
5. When automating, consider excess volumes required for setting up the instrument and dead volume of
robot pipette.
6. Ensure that no cross-contamination occurs between wells. Keep all pipettes and other equipment used
for Conjugate completely separate from the TMB Substrate reagent.
7. When pipetting Conjugate or TMB Substrate, aliquots for the required numbers of wells should be
taken to avoid multiple entry of pipette tips into the reagent bottles. Never pour unused reagents back
into the original bottles.
8. Do not allow microwells to dry between incubation steps.
9. Strictly follow the described wash procedure. Insufficient washing may cause high background signal.
10. Avoid direct sunlight and exposure to heat sources during all incubation steps.
11. Replace colour-coded caps on their correct vials to avoid cross-contamination
12. It is important to dispense all samples and controls into the wells without delay. Therefore ensure that
all samples are ready to dispense.

7. Shelf life and storage conditions

On arrival, store the kit at 2 – 8°C. Once opened the kit is stable for 3 months (or until its expiry date if less
than 3 months). Do not use kits beyond their expiry date. Do not freeze any kit component. The diluted
Wash Buffer has a shelf life of 3 months if stored in a closed bottle at 2 – 8°C

8. Specimen collection and storage

Serum or plasma samples may be used and should be stored at -20°C for long-term storage. Frozen samples
must be mixed well after thawing and prior to testing. Repeated freezing and thawing can affect results.
Addition of preservatives to the serum sample may adversely affect the results. Microbiologically
contaminated, heat-treated or specimens containing particulate matter should not be used. Grossly haemolysed, icteric or
lipaemic specimens should be avoided.
9. Preparation of reagents

1. Dilute the Wash Buffer (Reagent 2) 1:14 in distilled water to make sufficient buffer for the assay run.

10. Assay Procedure

1. Dilute patient samples 1:50 in diluted Sample Diluent (e.g. 10µl serum plus 0.5ml diluent).
2. Assemble the number of strips required for the assay.
3. Dispense 100 µl of each Standard, the Negative and Positive Controls and the diluted patient samples into appropriate wells.
4. Incubate for 30 minutes at room temperature.
5. After 30 minutes, decant or aspirate the well contents and wash the wells 3 times using automated washing or the manual wash procedure (see below). Careful washing is the key to good results. **Do not allow the wells to dry out.**
   
   **Manual Wash Procedure:**
   Empty the wells by inversion. Using a multi-channel pipette or wash bottle, fill the wells with wash buffer. Empty by inversion and blot the wells on absorbent paper. Repeat this wash process 2 more times.
6. Dispense 100µl of Conjugate (Reagent 3) into each well. Incubate the wells for 30 minutes at room temperature.
7. After 30 minutes, discard the well contents and carefully wash the wells 4 times with Wash Buffer. Ensure that the wells are empty but do not allow to dry out.
8. Using a repeating dispenser, rapidly dispense 100µl of TMB Substrate (Reagent 4) into each well. Incubate the plate for 10 minutes.
9. Add 100µl of Stop Solution (Reagent 5) to each well. To allow equal reaction times, the Stop Solution should be added to the wells in the same order as the TMB Substrate.
10. Read the optical density (OD) of each well at 450nm in a microplate reader within 10 minutes. A 620nm filter may be used as a reference wavelength.

11. Quality control

Quality control data is supplied on the lot-specific QC certificate included in the kit.

Controls are intended to monitor for substantial reagent failure. Any well positive by spectrophotometer but without visible colour should be cleaned on the underside and re-read. If OD values below zero are observed, the wavelengths used should be verified, the reader re-blanked to air and the measurements repeated.

12. Interpretation of Results

Plot the OD of each standard against its concentration and draw the best-fit curve through the points. Read the unknowns off this curve.

Samples with concentrations < 10 U/ml are considered negative.
Samples with concentrations >10 U/ml are considered positive.

13. Limitations of the Procedure

For diagnostic purposes, prothrombin IgG ELISA results should be used in conjunction with other test results and overall clinical presentation.

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14. Clinical studies

Disease populations
In a study of 40 patients with systemic rheumatic disease, including systemic lupus erythematosus, Sjögren’s syndrome, CREST and mixed connective tissue disorder, 26 were positive for anti-prothrombin IgG activity (65%). Range 1.3 – 91 U/ml. All patients were positive for either anti-dsDNA IgG or anti-nuclear antibodies. All patients were negative for anti-β2-glycoprotein 1 IgG.

Of 20 patients with APS, 9 (45%) were positive for anti-prothrombin IgG. Range 3.1 – 42.3 U/ml. All patients were positive for anti-β2-glycoprotein 1 IgG.

Control subjects
In a study of 72 healthy control subjects, 95% of subjects had anti-prothrombin activity below 10 U/ml. Range: 1.3 – 21.0 U/ml; median: 3.7 U/ml.

15. Reproducibility

Within Assay Imprecision < 12%
Between Assay Imprecision < 12%

Method Summary

- Dilute sera 1:50 with Sample Diluent (Reagent 1)
- Dispense Standards, the Positive and Negative Controls and the diluted sample into the microplate wells
- Incubate for 30 minutes at room temperature.
- Wash the wells three times
- Dispense 100µl of Conjugate (Reagent 3) into each well
- Incubate at room temperature for 30 minutes
- Wash the wells four times
- Add 100µl of TMB Substrate (Reagent 4) to each well
- Incubate at room temperature for 10 minutes
- Add 100µl Stop Solution (Reagent 5) to each well
- Read the optical density at 450nm (single wavelength) or 450/620nm (dual wavelength).

Further reading

Akimoto T et al: Relationship between clinical features and binding domains of anti-prothrombin autoantibodies in patients with systemic lupus erythematosus and antiphospholipid syndrome. Lupus 1999; 761-6