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Instruction for use
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ORG 541 Anti-Prothrombin IgG / IgM

Immunometric Enzyme Immunoassay for the quantitative determination of Anti-Prothrombin (IgG and IgM) antibodies

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NAME AND INTENDED USE

Anti-Prothrombin IgG / IgM is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG and IgM class autoantibodies against Prothrombin in human serum or plasma. The assay is intended for in vitro diagnostic use only as an aid in the diagnosis of an increased risk of thrombosis in patients with Systemic Lupus Erythematosus (SLE) or lupus-like disorders.

SUMMARY AND EXPLANATION OF THE TEST

Anti-phospholipid (aPL) antibodies have been associated with venous and arterial thrombosis as well as with recurrent fetal loss. Patients with these symptoms and high aPL antibody levels have been diagnosed as having the anti-phospholipid antibody syndrome (APS). Anti-phospholipid syndrome can occur in patients with systemic lupus erythematosus (SLE) or other autoimmune diseases (secondary anti-phospholipid syndrome) or in patients without underlying autoimmune disease (primary anti-phospholipid syndrome).

In clinical practice anti-cardiolipin antibodies detected by ELISA is one of the most established and standardised tests for diagnosis of the antiphospholipid syndrome. However, the family of aPL antibodies has recently expanded to include a heterogeneous group of antibodies whose specificity is directed against phospholipid binding proteins or their complex with phospholipids.

Among the phospholipid binding proteins, the best studied is β_2 -glycoprotein 1 (β_2 GP1). Another phospholipid binding protein, Prothrombin (factor II), exerts a procoagulant activity via a prothrombinase complex, triggering fibrinogen conversion to fibrin.

Autoantibodies can inhibit the formation of the thrombokinase complex. This complex is formed by the binding of prothrombin to phospholipids on the damaged cell membrane where prothrombin, then, is enzymatically transformed into thrombin. Thrombin, itself, causes blood coagulation. Antibodies directed against coagulation factors, like prothrombin, are pathogenic antibodies, that circulate in the blood stream. They directly inhibit the coagulation factors, and hence prolong the coagulation time. These autoantibodies are also found in association with autoimmune diseases.

The presence of antibodies to prothrombin is associated with thrombosis in patients with SLE and APS. And it was found that high autoantibody levels against prothrombin imply a risk of deep venous thrombosis and pulmonary embolism and could be involved in the development of the thrombotic processes. Furthermore, it was demonstrated that elevated levels of antibodies to prothrombin predict a risk of myocardial infarction.

PRINCIPLE OF THE TEST

Highly purified Prothrombin is bound to microwells. Antibodies against this antigen, if present in diluted serum or plasma, bind to the respective antigen. Washing of the microwells removes unspecific serum and plasma components. Horseradish peroxidase (HRP) conjugated anti-human IgG or IgM immunologically detect the bound patient antibodies forming a conjugate/antibody/antigen complex. Washing of the microwells removes unbound conjugate. An enzyme substrate in the presence of bound conjugate hydrolyzes to form a blue color. The addition of an acid stops the reaction forming a yellow end-product. The intensity of this yellow color is measured photometrically at 450 nm. The amount of colour is directly proportional to the concentration of IgG resp. IgM antibodies present in the original sample.

WARNINGS AND PRECAUTIONS

1. All reagents of this kit are strictly intended for in vitro diagnostic use only.
2. Do not interchange kit components from different lots.
3. Components containing human serum were tested and found negative for HBsAg, HCV, HIV1 and HIV2 by FDA approved methods. No test can guarantee the absence of HBsAg, HCV, HIV1 and HIV2, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
4. Avoid contact with the TMB (3,3',5,5'-Tetramethyl-benzidine). If TMB comes into contact with skin, wash thoroughly with water and soap.
5. Avoid contact with the Stop Solution which is hydrochloric acid (1 M). If it comes into contact with skin, wash thoroughly with water and seek medical attention.
6. Some kit components (i.e. Controls, Sample buffer and Buffered Wash Solution) contain Sodium Azide as preservative. Sodium Azide (NaN_3) is highly toxic and reactive in pure form. At the product concentrations (0,09%), though not hazardous. Despite the classification as non-hazardous, we strongly recommend using prudent laboratory practices (see 8., 9., 10.).
7. Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush drains with copious amounts of water to dilute the components below active levels.
8. Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
9. Do not pipette by mouth.
10. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
11. Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme temperature may initiate spontaneous combustion.

Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all kit reagents, controls and serum samples observe the existing legal regulations.

CONTENTS OF THE KIT

Package size	96 determ.
Qty.1	Divisible microplate consisting of 12 modules of 8 wells each, coated with highly purified Prothrombin. Ready to use.
6 vials, 1.5 ml each	combined Calibrators with IgG and IgM class Anti-Prothrombin antibodies (A-F) in a serum/buffer matrix (PBS, BSA, NaN_3 <0,1% (w/w)) containing: 0; 6.3; 12.5; 25; 50; and 100 U/ml. Ready to use.
2 vials, 1,5 ml each	Anti-Prothrombin Controls in a serum/buffer matrix (PBS, BSA, NaN_3 <0,1% (w/w)) positive (1) and negative (2), for the respective concentrations see the enclosed package insert. Ready to use.
1 vial, 20 ml	Sample buffer (Tris, NaN_3 <0,1% (w/w)), yellow, concentrate (5x).

1 vial, 15 ml	Enzyme conjugate solution (PBS, PROCLIN 300 <0,5% (v/v)), (light red) containing polyclonal rabbit anti-human IgG; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 ml	Enzyme conjugate solution (PBS, PROCLIN 300 <0,5% (v/v)), (light red) containing polyclonal rabbit anti-human IgM; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 ml	TMB substrate solution. Ready to use.
1 vial, 15 ml	Stop solution (1 M hydrochloric acid). Ready to use.
1 vial, 20 ml	Wash solution (PBS, NaN_3 <0,1% (w/w)), concentrate (50x).

STORAGE AND STABILITY

1. Store the kit at 2-8 °C.
2. Keep microplate wells sealed in a dry bag with desiccants.
3. The reagents are stable until expiration of the kit.
4. Do not expose test reagents to heat, sun or strong light during storage and usage.
5. Diluted sample buffer and wash buffer are stable for at least 30 days when stored at 2-8 °C.

MATERIALS REQUIRED

Equipment

- Microplate reader capable of endpoint measurements at 450 nm
- Multi-Channel Dispenser or repeatable pipet for 100 μl
- Vortex mixer
- Pipets for 10 μl , 100 μl and 1000 μl
- Laboratory timing device
- Data reduction software

Preparation of reagents

- Distilled or deionized water
- Graduated cylinder for 100 and 1000 ml
- Plastic container for storage of the wash solution

SPECIMEN COLLECTION, STORAGE AND HANDLING

1. Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
2. Allow blood to clot and separate the serum by centrifugation.
3. Test serum should be clear and non-hemolyzed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
4. Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months.

- Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity.
- Testing of heat-inactivated sera is not recommended.

PROCEDURAL NOTES

- Do not use kit components beyond their expiration dates.
- Do not interchange kit components from different lots.
- All materials must be at room temperature (20-28 °C).
- Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
- Perform the assay steps only in the order indicated.
- Always use fresh sample dilutions.
- Pipette all reagents and samples into the bottom of the wells.
- To avoid carryover contamination change the tip between samples and different kit controls.
- It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
- All incubation steps must be accurately timed.
- Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
- Do not re-use microplate wells.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

PREPARATION OF REAGENTS

Preparation of sample buffer

Dilute the contents of each vial of the sample buffer concentrate (5x) with distilled or deionized water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of wash solution

Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled or deionized water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2-8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Sample preparation

Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well. Controls are ready to use and need not be diluted.

TEST PROCEDURE

- Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
- Pipet **100 µl** of calibrators, controls and prediluted patient samples in duplicate into the wells. For determination of both IgG and IgM autoantibodies calibrators, controls and patient samples have to be pipetted in two attempts.

	1	2	3	4	5	6
A	SA	SE	P1	P5		
B	SA	SE	P1	P5		
C	SB	SF	P2	P..		
D	SB	SF	P2	P..		
E	SC	C1	P3			
F	SC	C1	P3			
G	SD	C2	P4			
H	SD	C2	P4			

SA-SF: standards A to F
P1, P2...C: patient sample 1, 2 ...
C1: positive control
C2: negative control

- Incubate for 30 minutes at room temperature (20-28 °C).
- Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
- Dispense **100 µl** of enzyme conjugate into each well.
- Incubate for 15 minutes at room temperature.
- Discard the contents of the microwells and wash 3 times with **300 µl** of wash solution.
- Dispense **100 µl** of TMB substrate solution into each well.
- Incubate for 15 minutes at room temperature.
- Add **100 µl** of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
- Read the optical density at 450 nm and calculate the results. Bi-chromatic measurement with a reference at 600-690 nm is recommended.

The developed colour is stable for at least 30 minutes. Read optical densities during this time.

Automation

The ORGENTEC Anti-Prothrombin IgG / IgM ELISA is suitable for use on open automated ELISA processors. The test procedure detailed above is appropriate for use with or without automation.

INTERPRETATION OF RESULTS

Quality Control

This test is only valid if the optical density at 450 nm for Positive Control (1) and Negative

Control (2) as well as for the Calibrator A and F complies with the respective range indicated on the Quality Control Certificate enclosed to each test kit ! If any of these criteria is not fulfilled, the results are invalid and the test should be repeated.

Calculation of results

For Anti-Prothrombin ELISA a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice.

Recommended Lin-Log Plot

First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

Calculation example

The figures below show typical results for Anti-Prothrombin IgG/IgM ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

Calibrators									
No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl.Conc.	CV%
ST1	A 1/A 2	0.038	0.039	0.038	0,001	0,001	0,001	0,001	2
ST2	B 1/B 2	0.227	0.232	0.229	7.1	7.3	7.2	6.3	1
ST3	C 1/C 2	0.388	0.395	0.391	12.6	12.8	12.7	12.5	1
ST4	D 1/D 2	0.744	0.764	0.754	25.0	25.7	25.3	25	2
ST5	E 1/E 2	1.336	1.306	1.321	49.0	47.7	48.3	50	1
ST6	F 1/F 2	2.209	2.226	2.218	100.8	102.1	101.4	100	0

Interpretation of results

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Anti-Prothrombin tests:

	Anti-Prothrombin-Ab	
	IgG [U/ml]	IgM [U/ml]
normal:	< 10	<10
elevated:	≥ 10	≥ 10

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually. It is recommended that each laboratory establishes its own normal and pathological ranges of serum Anti-Prothrombin. The values below should be regarded as guidelines only.

PERFORMANCE CHARACTERISTICS

Parallelism

In dilution experiments sera with high IgG and IgM class antibody concentrations were diluted with sample buffer and assayed in the Anti-Prothrombin kit. The assay showed linearity over the full measuring range.

Precision (Reproducibility)

Statistics for coefficients of variation (CV) were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 5 different runs with 6 determinations each:

anti-Prothrombin (IgG)			anti-Prothrombin (IgM)		
Intra-Assay			Intra-Assay		
Sample No	Mean [U/ml]	CV [%]	Sample No	Mean [U/ml]	CV [%]
1	9.5	3.8	1	13.7	3.9
2	18.3	4.2	2	23.7	4.2
3	31.9	3.3	3	42.9	4.0
Inter-Assay			Inter-Assay		
Sample No	Mean [U/ml]	CV [%]	Sample No	Mean [U/ml]	CV [%]
1	10.4	9.3	1	14.4	8.0
2	19.0	5.2	2	24.8	5.9
3	34.1	6.0	3	45.5	8.2

Sensitivity

The lower detection limit for Anti-Prothrombin IgG and IgM was determined at 1.0 U/ml.

Specificity

The microplate is coated with highly purified Prothrombin. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of Prothrombin after immobilisation on the solid phase. The Anti-Prothrombin test kits are specific only for autoantibodies directed against Prothrombin.

Calibration

Since no international reference preparation for anti-prothrombin autoantibodies is available, the assay system is calibrated in relative arbitrary units.

LIMITATIONS OF PROCEDURE

The Anti-Prothrombin IgG/IgM ELISA is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated.

INTERFERING SUBSTANCES

No interference has been observed with haemolytic (up to 1000 mg/dL), lipemic (up to 3 g/dL triglycerides) or bilirubin (up to 40 mg/dL) containing sera. Nor have any interfering effects been observed with the use of anticoagulants. However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

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INCUBATION SCHEME

