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### 1 NAME AND INTENDED USE

Anti-Annexin V IgG/IgM is an indirect solid phase enzyme immunoassay (ELISA) for the quantitative measurement of IgG and IgM class autoantibodies against Annexin V in human serum or plasma

The assay is intended for in vitro use only as an aid in the determination of an increased risk of thrombosis and recurrent fetal loss. In the United States, this kit is intended for Research Use Only.

### 2 SUMMARY AND EXPLANATION OF THE TEST

Anti-phospholipid binding protein antibodies represent a family of immunoglobulins which recognise phospholipids, alone or bound to plasma protein cofactor(s), or the cofactors them-selves (1). A variety of proteins have been implicated including: prothrombin, B2-glycoprotein I, protein S and annexin V (13). Autoimmune anti-phospholipid-protein antibodies are associa-ted with a variety of thromboembolic complications involving both arterial and venous sites, as well as recurrent miscarriages and thrombocytopenia (3). The pathophysiologic mecha-nisms of this antiphospholipid syndrome (APS) have not vet been identified (10).

Annexin V, also known as placental anticoagulant protein I (PAP I) (8), not only belongs to a family of proteins that bind to phospholipids in a calcium dependent manner (5), but is also a potent vascular anticoagulant protein (9). Annexin V is necessary to maintain placental integri-ty and may have a thromboregulatory effect at the materno-fetal interface as shown in a muri-ne model (14).

It has been suggested, that interference of anti-annexin V antibodies with the annexin V shield on the placental villi leads to the exposure of anionic procoagulant phospholipids. Anionic procoagulant phospholipids are known to enhance blood coagulation processes in the pla-cental vasculature, and may be an important contributory factor of thrombosis and recurrent loss of pregnancy in the APS (9).

Anti-annexin V antibodies are not only highly specific for APS, but also elevated levels of anti-bodies are detected in patients with systemic lupus diseases (SLE) (4, 6).

It has been suggested that anti-annexin V autoantibodies may influence in the clinical course of rheumatoid arthritis (2, 11). A significantly high frequency of arterial or venous thrombosis is found in patients with anti-annexin V antibodies (12).

And are a significant risk factor for recurrent miscarriages in SLE patients (7).

### **PRINCIPLE OF THE TEST** 3

Human Annexin V 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 and 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.





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### WARNINGS AND PRECAUTIONS 4

- 1. All reagents of this kit are strictly intended for in vitro use only. In the United States, this kit is intended for Research 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 or HIV2, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.
- Avoid contact with the TMB (3.3', 5.5'-Tetramethyl-benzidine). If TMB comes into contact with skin, wash 4. thoroughly with water and soap.
- Avoid contact with the Stop Solution which is acid. If it comes into contact with skin, wash thoroughly with water 5. and seek medical attention.
- Some kit components (i.e. Controls, Sample buffer and Buffered Wash Solution) contain Sodium Azide as 6. preservative. Sodium Azide (NaN<sub>3</sub>) 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.)
- Some kit components contain Proclin 300 as preservative. When disposing reagents containing Proclin 300, flush 7. 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** 5

Package size 96 determ.

Qty.1	Divisible <b>microplate</b> consisting of 12 modules of 8 wells each, coated with human Annexin V. Ready to use.					
6 vials, 1.5 mL each	combined <b>Standards</b> with IgG and IgM class Anti-Annexin V (A-F) in a serum/buffer matrix (PBS, BSA, NaN <sub>3</sub> <0.1% (w/w): 0; 6.3; 12.5; 25; 50;and 100 U/mL. Ready to use.					
3 vials, 1.5 mL each	Anti-Annexin V <b>Controls</b> in a serum/buffer matrix (PBS, BSA, NaN <sub>3</sub> <0.1% (w/w)) positive on IgG (1), positive on IgM (2)and negative on IgG and IgM (3). Ready to use.					

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1 vial, 20 mL	Sample buffer (Tris, NaN <sub>3</sub> <0.1% (w/w)), yellow, concentrate (5x).
1 vial, 15 mL	<b>Enzyme conjugate</b> solution (PBS, Proclin 300 <0.5% (v/v)), (light red) containing polyclonal rabbit <b>anti-human IgG</b> ; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 mL	<b>Enzyme conjugate</b> solution (PBS, Proclin 300 <0.5% (v/v)), (light red) containing polyclonal rabbit <b>anti-human-IgM</b> ; labelled with horseradish peroxidase. Ready to use.
1 vial, 15 mL	TMB substrate solution. Ready to use.
1 vial, 15 mL	Stop solution (contains acid). Ready to use.
1 vial, 20 mL	Wash solution (PBS, NaN <sub>3</sub> <0.1% (w/w)), concentrate (50x).

### STORAGE AND STABILITY 6

- Store the kit at 2-8 °C. 1.
- 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

### 7 **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  $\mu$ L, 100  $\mu$ L and 1000  $\mu$ L
- 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

### 8 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.
- Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months. 4.

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- Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity. 5.
- Testing of heat-inactivated sera is not recommended. 6.

### **PROCEDURAL NOTES** 9

- 1. Do not use kit components beyond their expiration dates.
- 2. Do not interchange kit components from different lots.
- 3. 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 4. interruption to get the most reliable and consistent results.
- 5. Perform the assay steps only in the order indicated.
- 6. Always use fresh sample dilutions.
- 7. Pipette all reagents and samples into the bottom of the wells.
- 8. To avoid carryover contamination change the tip between samples and different kit controls.
- 9. It is important to wash microwells thoroughly and remove the last droplets of wash buffer to achieve best results.
- 10. All incubation steps must be accurately timed.
- 11. Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.
- 12. 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.

## **10 PREPARATION OF REAGENTS**

### **10.1** 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.

### **10.2** 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.

### **10.3** Sample preparation

Dilute all patient samples 1:100 with sample buffer before assay.

Therefore combine 10  $\mu$ L of sample with 990  $\mu$ L of sample buffer in a polystyrene tube. Mix well.







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Controls are ready to use and need not be diluted.

### **11 TEST PROCEDURE**

- 1. Prepare a sufficient number of microplate modules to accommodate controls and prediluted patient samples.
- Pipet 100 µL of standards, controls and prediluted patient samples in duplicate into the wells. 2.

	1	2	3	4	5	6		
А	SA	SE	P1	P5				
В	SA	SE	P1	P5				
С	SB	SF	P2	Р				
D	SB	SF	P2	Р			SA-SF:	standards A to F
Е	SC	C1	P3				P1, P2C:	patient sample 1, 2
F	SC	C1	P3				C1:	positive control
G	SD	C2	P4				C2:	negative control
Н	SD	C2	P4					

- 3. Incubate for 30 minutes at room temperature (20-28 °C).
- 4. Discard the contents of the microwells and wash 3 times with  $300 \,\mu\text{L}$  of wash solution.
- 5. Dispense  $100 \,\mu L$  of enzyme conjugate into each well.
- Incubate for 15 minutes at room temperature. 6
- 7. Discard the contents of the microwells and wash 3 times with 300  $\mu$ L of wash solution.
- 8. Dispense 100 µL of TMB substrate solution into each well.
- 9. Incubate for 15 minutes at room temperature.
- 10. Add 100  $\mu$ L of stop solution to each well of the modules and incubate for 5 minutes at room temperature.
- 11. 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 Anti-Annexin V 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.





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## **12 INTERPRETATION OF RESULTS**

## 12.1 Quality Control

This test is only valid if the optical density at 450 nm for Positive Control (1, 2) and Negative Control (3) as well as for the Standard 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.

### 12.2 Calculation of results

For Anti-Annexin V IgG and IgM 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 standard well. Use lin-log graph paper and plot the averaged optical density of each standard versus the concentration. Draw the best fitting curve approximating the path of all standard points. The standard 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-Annexin V IgG/IgM ELISA. These data are intended for illustration only and should not be used to calculate results from another run.

Standards									
No	Position	OD 1	OD 2	Mean	Conc. 1	Conc. 2	Mean	decl. Conc.	CV %
STA	A 1/B 1	0.076	0.074	0.075	0.09	0.09	0.09	0	1.9
STB	C 1/D 1	0.286	0.288	0.287	5.6	5.5	5.6	6.3	0.5
STC	E 1/F 1	0.610	0.629	0.620	12.0	12.4	12.2	12.5	2.2
STD	G 1/H 1	1.199	1.223	1.211	25.8	26.5	26.2	25	1.4
STE	A 2/B 2	1.803	1.743	1.773	49.3	46.2	47.7	50	2.4
STF	C 2/D 2	2.381	2.318	2.350	109.8	98.0	103.9	100	1.9

## **Interpretation of results**

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

<u>Anti-Annexin V</u>	IgG and IgM
negative:	< 5 U/mL
borderline	5 - 8 U/mL
positive	> 8 U/mL

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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-Annexin V. The values below should be regarded as guidelines only.

## **PERFORMANCE CHARACTERISTICS**

## Parallelism

In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the Anti-Annexin V kit. The assay shows 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 6 different runs with 8 determinations each:

Intra-Assay						
Sample	Mean	CV				
No	[U/ml]	[%]				
1	11	6.7				
2	27	3.8				
3	67	4.2				

Inter-Assay						
Sample	Mean	CV				
No	[U/ml]	[%]				
1	9	4.1				
2	23	4.2				
3	64	5.7				

### Sensitivity

The lower detection limit for Anti-Annexin V IgG and IgM was determined at 1.0 U/mL.

### Specificity

The microplate is coated with human Annexin V. The Anti-Annexin V test kit recognises only autoantibodies specific to the Annexin V.

### Calibration

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











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## LIMITATIONS OF PROCEDURE

The Anti-Annexin V IgG/IgM ELISA is a diagnostic aid and by itself is not diagnostic. 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 be avoided.

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# **RUO** IN THE USA

### SYMBOLS USED WITH DRG ASSAYS

Symbol	English	Deutsch	Français	Español	Italiano
[]i	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts- kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
Σ	Contains sufficient for <n> tests/</n>	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos</n>	Contenuto sufficiente per "n" saggi
<b>1</b>	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
$\square$	Expiration Date	Mindesthaltbarkeits- datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità
Symbol	Portugues	Dansk	Svenska	Ελληνικά	
[]i	Consulte as instruções de utilização	Se brugsanvisning	Se bruksanvisningen	Εγχειρίδιο χρήστη	
CE	Conformidade com as normas europeias	Europaeisk overensstemmelse	Europeisk överensstämmelse	Ευρωπαϊκή Συμμόρφωση	
IVD	Diagnóstico in vitro	In vitro diagnostik	Diagnostik in vitro	in vitro διαγνωστικό	
RUO					
REF	Catálogo n.º	Katalognummer	Katalog nummer	Αριθμός καταλόγου	
LOT	No do lote	Lot nummer	Batch-nummer	Αριθμός Παρτίδος	
Σ		Indeholder tilsttrækkeligt til "n" test	Innehåller tillräckligt till "n" tester	Περιεχόμενο επαρκές για «n» εξετάσεις	
<b>1</b>	Temperatura de conservação	Opbevarings-temperatur	Förvaringstempratur	Θερμοκρασία αποθήκευσης	
$\Sigma$	Prazo de validade	Udløbsdato	Bäst före datum	Ημερομηνία λήξης	
***	Fabricante	Producent	Tillverkare	Κατασκευαστής	
Distributed by					
Content	Conteúdo	Indhold	Innehåll	Περιεχόμενο	
Volume/No.	Volume/Número	Volumen/antal	Volym/antal	Όγκος/αριθ	