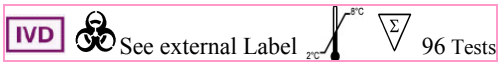
Diagnostic Automation / Cortez Diagnostics, Inc.
IMMUNO DIAGNOSTICS

AccuDiag™ Cardiolipin IgG/IgA/IgM ELISA Kit

REF 1490-1



Test	Cardiolipin IgG/IgA/IgM ELISA
Method	Enzyme Linked Immunosorbent Assay
Principle	Indirect; Antigen Coated Plate
Detection Range	Qualitative Positive, Negative Control
Sample	5 µL serum
Total Time	~ 90 min.
Shelf Life	12 Months from the manufacturing date

INTENDED USE

The Diagnostic Automation Inc, Cardiolipin IgG/IgA/IgM (ACA Screen) ELISA kit is an enzyme linked immune-assay (ELISA) for the qualitative detection of Cardiolipin IgA, IgG and IgM antibodies in human serum to aid in the diagnosis of anti-phospholipid syndrome (APS) and APS associated with systemic lupus erythematosus (SLE) in conjunction with other laboratory tests and clinical findings.

Warning: Confirmed active or seropositive syphilis patients can have elevated anti-cardiolipin antibody (ACA) levels. To rule out syphilis, confirmatory tests should be performed.

SUMMARY AND EXPLANATION

Antiphospholipid antibodies are a heterogeneous group of autoantibodies against negatively charged phospholipids.1 They are detected primarily by the anti-cardiolipin antibody (ACA) test, the biological false positive test for syphilis and the lupus anticoagulant test. These three tests detect related, but not necessarily identical antibodies. Thus, more than one of these tests is sometimes necessary to identify antiphospholipid antibodies. The ELISA assay format is a highly sensitive method for detection of antiphospholipid antibodies.[2]

The presence of anti-cardiolipin antibodies helps to identify patients at risk of venous and/or arterial thrombosis often accompanied by thrombocytopenia, a syndrome referred to as antiphospholipid syndrome.[1-12] The antiphospholipid syndrome most commonly occurs in patients with systemic lupus erythematosus (SLE) or lupus-like disease where the criteria for SLE are not fulfilled.[5-7] High levels of anti-cardiolipin antibodies occur in thrombosis, fetal loss, thrombocytopenia and several other disorders.[1-15] Low levels of anti-cardiolipin antibodies are found in a variety of clinical disorders which are unrelated to antiphospholipid syndrome. Therefore, low levels of these antibodies are of limited significance.

IgG and IgA class anti-cardiolipin antibodies appear to be more closely associated with antiphospholipid syndrome than the IgM class antibodies. However, IgM antibodies appear to be more influenced by treatment.[5,10] Low levels of IgM antibodies can be identified in other autoimmune diseases such as rheumatoid arthritis, primary Sjögren's Syndrome, drug induced lupus erythematosus, Lyme disease, and syphilis.[8,10]

TEST PRINCIPLE

Cardiolipin antigen as well as bovine and human B2GP1 is bound to the wells of a polystyrene microwell plate followed by blocking the unreacted sites to reduce non-specific binding. Controls, calibrator and diluted patient sera are added to separate wells, allowing any cardiolipin antibodies present to bind to the immobilized antigen. Unbound sample is washed away and an enzyme labeled anti-human IgA/IgG/IgM conjugate is added to each well. These enzyme conjugated antibodies bind specifically to the human immunoglobulin of the appropriate class. After washing away any unbound conjugate, specific enzyme substrate (TMB) is then added to the wells. After stopping the enzymatic reaction, the intensity of color change, which is proportional to the concentration of antibody, is read by a spectrophotometer at 450 nm. Results are expressed in ELISA units per milliliter (EU/ml).

SPECIMEN COLLECTION AND PREPARATION

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples. It is recommended that frozen specimens be tested within one year.

PRECAUTIONS

All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. However, human blood derivatives and patient specimens should be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials.[16]

Stop Solution is a dilute sulfuric acid solution. Sulfuric acid (H2SO4) is poisonous and corrosive. Do not ingest and avoid contact with skin and eyes. Avoid exposure to bases, metals or other compounds that may react with acids.

TMB Enzyme Substrate contains an irritant that may be harmful if inhaled, ingested or absorbed through the skin. Do not ingest and avoid contact with skin and eyes.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use kit components beyond expiration date on the labels.

MATERIALS AND COMPONENTS

Materials provided with the test kits

1. **Plate:** Microplate with individual breakaway microwells. Coated with cardiolipin antigen and bovine/human B2GP1. Ready for use.
2. **Positive Control:** 1 x 1.75 ml. Ready to use *(red cap)*. Contains human serum positive for ACA. The expected concentration range in EU/ml is printed on the label.
3. **Negative Control:** 1 x 1.75 ml. Ready to use *(white cap)*. Contains human serum.
4. **Calibrator:** 1 x 1.75 ml. Ready to use Calibrator (green cap) 30 EU/ml. Derived from human serum containing ACA.
5. **Conjugate:** 1 x 15 ml. HRP goat anti-human IgA/IgG/IgM Conjugate. Ready for use. Color coded pink.
6. **Diluent:** 1 x 60 ml. Serum Diluent. Ready for use.

7. **Substrate** TMB: 1 x 15 ml. TMB enzyme substrate. Ready for use. **Protect from light**.
8. **Stop Solution:** 1 x 15 ml. H_2SO_4,1X15 ml. Ready for use.
9. **Wash Buffer:** 2x vials. Powder Wash Buffer. **Reconstitute to one liter each**.
10. **Protocol Sheets**

Optional Components
1x60 ml Wash Buffer – Liquid concentrated Wash Buffer. **Reconstitute to one liter each**.

Materials required but not provided
1. Deionized or distilled water
2. Squeeze bottle to hold diluted wash buffer
3. Pipettes capable of delivering 5 µl to 1000 µl
4. Disposable pipette tips
5. Clean test tubes 12 x 75 mm and test tube rack
6. Timer
7. Absorbent paper towels
8. Microplate reader capable of reading absorbance values at 450 nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm
9. Automatic microplate washer capable of dispensing 200 µl

Procedural Notes

1. Carefully read the product insert before starting the assay.
2. Let patient specimens and test reagents equilibrate to room temperature before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
3. Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.
4. All dilutions of the patient samples should be prepared prior to starting with the assay.
5. Good washing technique is critical. If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. An automated microplate washer is recommended.
6. Use a multichannel pipette capable of delivering 8 or 12 wells simultaneously. This speeds the process and provides more uniform incubation times.
7. For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
8. Addition of all samples and reagents should be performed at the same rate and in the same sequence.

ASSAY PROCEDURE

Step 1 Let all reagents and specimens equilibrate to room temperature.
Step 2 Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.
Step 3 It is recommended that samples be dispensed according to the layout below.

A	Blank	S5	Etc.
B	-	S6	
C	+	S7	
D	Cal	S8	
E	S1	S9	
F	S2	S10	
G	S3	S11	
H	S4	S12	
	1	2	3

Step 4 Prepare a **1:101** dilution of the patient samples by mixing **5 µl** of the patient sera with **500ul** of Serum Diluent.
Step 5 Remove the required microwells from pouch and return unused strips in the sealed pouch to refrigerator. Securely place the microwells into the extra provided holder.
Step 6 Pipette **100 µl** of Ready to use Calibrator, Positive and Negative controls and diluted patient samples **(1:101)** to the appropriate microwells as per protocol sheet.
Note: Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank.
Step 7 Incubate **30 minutes** (± 5 min) at room temperature.
Step 8 Wash **4x** with wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper towels. For automatic washers, program the washer as per manufacturer's instructions.
Step 9 Pipette **100 µl** of Conjugate into microwells.
Step 10 Incubate **30 minutes** (± 5 min) at room temperature.
Step 11 Wash all microwells as in Step 8.
Step 12 Pipette **100 µl** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.
Step 13 Incubate **30 minutes** (± 5 min) at room temperature.
Step 14 Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the Enzyme Substrate. Read absorbance values within 30 minutes of adding Stop Solution.
Step 15 Read absorbance of each microwell at **450 nm** using a single or at 450/630nm using a dual wavelength microplate reader against the reagent blank set at zero absorbance.

RESULTS

Calculations
The concentrations of the patient samples can be determined using the following formula:

QUALITATIVE DETERMINATION

Abs. of Test Sample
---------- X EU/ml of Calibrator = EU/ml Test Sample
Abs. of Calibrator

It is recommended that qualitative results be reported as "positive" or "negative." Sample results greater than or equal to 20 EU/ml are considered positive.

Calibrator
The Ready to Use Calibrator included must be used with each run.

Interpretation
Interpretation values were determined by testing 64 normal blood donors and non-antiphospholipid syndrome disease control specimens. The mean of the normal subjects plus 2.75 SD was established as the assay cutoff and assigned an arbitrary value of 20 EU/ml. The following information serves only as a guide in the interpretation of the laboratory results. Each laboratory must determine its own normal values.

ACA value Interpretation

≤20 EU/ml Negative
≥20 EU/ml Positive

The literature suggests that, low positive anti-cardiolipin antibody levels may occur in a variety of clinical disorders unrelated to antiphospholipid antibody syndrome. Hence according to the investigators recommendations the diagnosis of antiphospholipid antibody syndrome should be made only when the test results are moderately or highly positive1[4,19] Isotype-specific ACA assays are required to assess separate ACA IgA, IgG or IgM antibody levels. It is suggested that >40 GPL or MPL

units be employed to differentiate between low positive ACA levels unrelated to APS and moderate to high ACA levels associated with clinical manifestations of APS.20

PERFORMANCE CHARACTERISTICS

The utility of the Diagnostic Automation, Inc. ACA IgA/IgG/IgM Antibody ELISA was evaluated by testing well-characterized specimens from cardiolipin positive SLE subjects alongside disease controls. These specimens were also tested on commercially available ELISA kits for each isotype. Only specimens in the linear range of the assay were included in the method comparison. These results are summarized below.

A. Diagnostic Automation, Inc. ACA IgA/IgG/IgM Antibody ELISA vs. other ACA Screen ELISA:

DAI ACA Screen ELISA	Other ACA Screen ELISA			
		Positive	Negative	Total
	Positive	243	27	270
	Negative	36	299	335
	Total	279	326	605

Positive Percent Agreement: 87.1% (95% CI 80.5% - 90.7%)
Negative Percent Agreement: 91.7% (95% CI 88.0% - 94.4%)
Overall Percent Agreement: 89.6% (95% CI 86.8% - 91.9%)

Disease associated subjects (APS, SLE, APS with SLE, suspected APS and SLE): n=469
Disease controls: n=136

B. Cross Reactivity: A set of potentially cross-reactive specimens from individuals with other autoimmune disorders and infection known to cross-react with cardiolipin were tested for ACA using the ACA Screen ELISA.

Condition	n Tested	n Pos	% Pos
Celiac disease	18	0	0.0%
Mixed connective tissue disease	15	1	6.7%
Myositis	2	0	0.0%
Rheumatoid arthritis	85	9	10.6%
Sjögren's syndrome	20	2	10.0%
Syphilis	40	39	97.5%
Systemic sclerosis	36	2	5.6%
Thyroiditis	8	0	0.0%
Vasculitis	8	0	0.0%

Reproducibility
14 assays of samples in the low negative range, positive for ACA in the moderate positive range, near the cutoff and approximately +/- 20% of the assay cutoff were performed to determine qualitative reproducibility. Assay results for the negative, +20% and moderate positive specimens produced 100% qualitative agreement. Assay results for the approximately -20% specimen produced 97% qualitative agreement. Assay results for the near cutoff specimen produced 79% qualitative agreement.

Limit of Detection
Based on 60 replicates of the blank and 10 replicates each of 6 low-level (NHS) samples the limits of detection (LoD) for ACA antibodies was determined to be 5.2 EU/ml.

Interference
Interference was studied by mixing sera with known ACA levels with potentially interfering serum samples and studying deviation from expected results. No significant interference was demonstrated for the following substances at the levels indicated: Hemoglobin (2 g/L), Bilirubin (342 µmol/L), Rheumatoid Factor (100 EU/ml), Triglycerides (37 mmol/L), and Cholesterol (13 mmol/L).

QUALITY CONTROL

Calibrator, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.3. The Calibrator should have an absorbance reading of not less than 0.2; otherwise the test must be repeated. The negative control must be <10 EU/ml. If the test is run in duplicate, the mean of the two readings should be taken for determining EU/ml. The optical density of the Calibrator must be greater than that of the negative control and less than the absorbance of the positive control. The positive control should give values in the range stated on the vial.

LIMITATIONS OF THE PROCEDURE

The Diagnostic Automation, Inc. ACA Screen ELISA should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples. This method should be used for testing human serum samples only.

Testing for all three isotypes of ACA is strongly recommended. Testing for only one and not all isotypes may lead to false negative results. Furthermore, a diagnosis cannot be made on the basis of ACA results alone. The results of other laboratory tests and clinical findings must be considered. When a negative ACA test occurs in the presence of clinical indications, a lupus anticoagulant test or other additional testing is indicated. ACA also occur transiently in a variety of infectious diseases. In these cases patients positive for ACA should be retested following an appropriate interval. Confirmed active or seropositive syphilis patients can have elevated ACA levels. To rule out syphilis, confirmatory tests should be performed.[17]

Anti-cardiolipin antibodies have also been associated with neurological syndromes such as transient ischemic effects and migraine.13 In contrast, in patients with antiphospholipid syndrome, the antibodies usually persist for longer periods and may even precede the onset of clinical symptoms.[2-4]

EXPECTED VALUES

The incidence of ACA in various disease conditions is summarized in the following tables:
Incidence of ACA in SLE [15,18-19,21-24]

Antibody Isotype	SLE % Incidence	APS % Incidence
IgG	39-44	36-80
IgA	17-57	10-30
IgM	5-33	15-60
Any isotype	53	71

Disease Association of ACA [22,25-29]

Condition	% Incidence
RecurrentVenous Thrombosis	28-71
Recurrent Fetal Loss	28-64
Transverse Myelitis	50
Hemolytic Anemia	38
Thrombocytopenia	27-33
Arterial Occlusions	25-31
Livedo Reticularis	25
Pulmonary Hypertension	20-40

Sets of clinical samples were tested on the Diagnostic Automation, Inc. ACA Screen ELISA. Results demonstrating incidence in the populations for this study are provided below.

Category	N	Pos	%
APS with unknown ACA	90	56	62.2

status			
APS with SLE involvement and unknown ACA status	66	60	90.9
Sera submitted for APS testing	185	38	20.5
SLE	74	24	32.4
Sera submitted for SLE testing	88	16	18.2
Thrombocytopenia	15	2	13.3
Pre-eclampsia	15	3	20.0
Other AID *	192	14	7.3
NHS	158	3	1.9%

* Other autoimmune disease includes celiac disease, myositis, rheumatoid arthritis, scleroderma, thyroiditis and vasculitis.

STORAGE

Store all reagents at 2-8°C. Do not freeze. Reagents are stable until the expiration date when stored and handled as directed.

Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (20-25°C) prior to use.

Reconstitute the wash buffer to 1 liter with distilled or deionized water. When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date.

Coated microwell strips are for one time use only. Unused microwell strips should be carefully resealed in the pouch containing desiccants to prevent condensation and stored at 2-8°C.

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Diagnostic Automation/Cortez Diagnostics, Inc.
21250 Califa Street, Suite 102 and 116,
Woodland Hills, California 91367 USA

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