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See external label \downarrow 2°C-8°C



Σ = tests



cat. #1493-2

MICROWELL ELISA

Anti-Cardiolipin IgA

An Enzyme-Linked Immunosorbent Assay (ELISA) for
the detection of IgA Antibodies to Cardiolipin
Catalog No.1493-2

INTENDED USE

The Diagnostic Automation Inc. Cardiolipin Antibody Test System is an enzyme-linked immunosorbent assay (ELISA) designed for the semi-quantitative measurement of circulating IgA autoantibodies to cardiolipin. This test is for *in vitro* diagnostic use.

SIGNIFICANCE AND BACKGROUND

Autoantibodies directed against phospholipids, and anti-cardiolipin (aCL) in particular, have been associated with recurrent thrombosis, thrombocytopenia, and spontaneous abortions (1,2,3). aCL is observed in patients with systemic lupus erythematosus, in patients with other connective tissue disease (4), in individuals undergoing chlorpromazine treatment (5), as well as in persons who do not have chronic illness.

PRINCIPLE OF THE ELISA ASSAY

The Diagnostic Automation Inc. Cardiolipin IgA ELISA test system is designed to detect IgA class antibodies to Cardiolipin in human sera. The test procedure involves three incubation steps:

1. Test sera (properly diluted) are incubated in microwells coated with Cardiolipin antigen. Anti-Cardiolipin specific IgA antibodies in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.
2. Peroxidase conjugated goat anti-human IgA is added to the wells and the plate is incubated. The conjugate will react with antibody immobilized on the solid phase in step 1. The wells are washed to remove un-reacted conjugate.
3. The microtiter wells containing immobilized peroxidase conjugate are incubated with peroxidase substrate solution. Hydrolysis of the substrate by peroxidase produces a color change. After a period of time, the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the test sample.

KIT COMPONENTS

Reactive Reagents

1. Twelve, 1 x 8-well strips coated with Cardioliipin antigen from bovine heart. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
 2. Horseradish peroxidase conjugated goat anti-human IgA. Ready to use. One 15mL vial.
 3. Human positive serum control. One 0.5mL vial.
 4. Human positive calibrator. One 0.5mL vial.
 5. Human negative serum control. One 0.5mL vial.
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6. Sample diluent. One 25 mL bottle containing a PBS solution (pH 7.2 ± 0.2). Ready to use. (Green solution with a green cap; Product Number 005C). **NOTE:** This reagent may be used with any DAI ELISA test system utilizing Product Number 005C.
 7. Wash Buffer. One 100mL bottle containing phosphate-buffered-saline. 10X concentrate. **NOTE:** 1X solution will have a pH of 7.2 ± 0.2 . (Product Number: 0008C). **NOTE:** This reagent may be used with any DAI ELISA test system utilizing Product Number 0008C.

Reagents common to all Diagnostic Automation Inc. ELISA Test Systems

1. Substrate solution. One 12mL bottle containing 3,3',5,5'-tetramethylbenzidine (TMB). Ready to use
2. Stop solution. One vial containing 8.0mL of 1.0M H₂SO₄, 0.7M HCl. Ready to use. (Clear solution with a red cap).

Non-Reactive Components

1. Sample dilution plate. One 96 well microtiter plate for preparing serum dilutions.
2. Two mylar plate sealers.

PRECAUTIONS

1. For *In Vitro* Diagnostic Use.
2. The microwell strips do not contain any viable, infectious agents. However, the strips should be considered potentially infectious and handled accordingly. Wash solutions should be collected in a disposable basin and treated with 0.5% sodium hypochlorite (10% household bleach) at the end of the days run.
3. Do not use the ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
4. Wipe bottom of plate free of residual liquid and/or fingerprints which can alter optical density (OD) readings.
5. Control sera, conjugate, sample diluent, and wash buffer contain preservative which may be toxic if ingested; Thimerosal at a concentration of 0.04% (w/v).
6. Dilution or adulteration of these reagents may result in loss of sensitivity.
7. Do not substitute reagents from kits with different lot numbers or from other manufacturers.
8. Each donor unit used in the preparation of the controls was found to be negative when tested by an FDA approved method for the presence of HBsAg, and for antibodies to HIV-1, HIV-2, and HCV.

WARNING - POTENTIAL BIOHAZARDOUS MATERIAL

Because no test method can offer complete assurance that human immunodeficiency virus, hepatitis B virus, or other infectious agents are absent, these

specimens/reagents, as well as patient samples, should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiology and Biomedical Laboratories", 1984, p.12-16, 3rd edition- 1993, and OSHA Standard for Bloodborne Pathogens (6).

9. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin or mucous membranes.
10. Avoid microbial contamination of reagents. Incorrect results may occur.
11. Cross contamination of reagents and/or samples could cause false results.
12. Contamination of the TMB substrate solution with conjugate or other oxidants will cause the solution to change color prematurely. Do not use substrate solution if it has begun to turn blue. To help eliminate the possibility of contamination, refer to Test Procedure, Section D.1.
13. Reusable glassware must be washed out and thoroughly rinsed free of all detergents.
14. Strict adherence to the specified time and temperature of incubations is essential for accurate results. All reagents must be brought to 20-25°C before starting the assay.
15. Improper washing will cause false positive or false negative results. Be sure to blot the plates free of any residual wash solution before adding conjugate or substrate solution. Do not allow the wells to dry out between incubations.
16. Do not allow the stop solution to contact skin or eyes. If contact occurs, immediately flush with water.
17. Caution: Liquid waste at acid pH should be neutralized before adding to sodium hypochlorite (bleach).
18. Avoid splashing or generation of aerosols.
19. Do not expose reagents to strong light during storage or incubation.
20. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
21. Do not allow the conjugate to come in contact with containers which may have previously contained a solution utilizing sodium azide as a preservative. Residual amounts of sodium azide may destroy the conjugate's enzymatic activity.
22. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this kit.

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of reading at a wavelength of 450nm.
2. Microliter pipettes capable of accurately delivering 10 and 200 μ L.
3. Adjustable multichannel pipette (50 - 200 μ L) for dispensing conjugate, substrate and stop solution.
4. Reagent reservoirs for multichannel pipettes.
5. Wash bottle or plate washing system.
6. Distilled or deionized water.
7. One liter graduated cylinder.
8. Serological pipette: 1, and 10 or 25 mL.
9. Disposable pipette tips.
10. Paper towels.
11. Timer with alarm capable of measuring to an accuracy of \pm 1 second.
12. Disposal basin and disinfectant, (Example: 0.5% sodium hypochlorite, 10% household bleach).

SPECIMEN COLLECTION

Only freshly drawn and properly stored blood sera obtained by approved aseptic venipuncture procedures should be used in this assay (7, 8). No anticoagulants or preservatives should be added. Avoid using hemolyzed, lipemic, or bacterially contaminated sera.

Store sample at room temperature for no longer than 8 hours. If testing is not performed within 8 hours, sera may be stored at 2-10° C for no longer than 48 hours. If delay in testing is anticipated, store test sera at -20°C or lower. Avoid multiple freeze/thaw cycles which may cause loss of antibody activity and give erroneous results.

STORAGE CONDITIONS

1. Store the unopened kit at 2-8°C.
2. Cardiolipin coated microwell strips: Extra strips should be immediately resealed with desiccant and returned to storage at 2-8°C. Strips are stable for 60 days after the envelope has been opened and properly resealed and the indicator remains blue.
3. Peroxidase conjugated goat anti-human IgA: Store at 2-8°C. DO NOT FREEZE.
4. Human control and calibrator sera: Store at 2-8°C.
5. TMB substrate solution: Store at 2-8°C.
6. Wash buffer: Store at 2-25° C. Stable for 30 days at 2-8°C after diluting to 1X, or 7 days when stored at room temperature.
7. Sample diluent: Store at 2-8°C.
8. Stop solution: Store at 2-25°C.

NOTE: All kit components are stable until the expiration date printed on the label provided the recommended storage conditions are strictly followed. Do not use beyond the expiration date.

PROCEDURE

PREPARATION OF REAGENTS

1. Wash Buffer: Dilute the 100mL of 10X concentrate with 900mL of distilled or deionized water. Mix thoroughly to dissolve any crystals that may be present.
2. Sample diluent, stop solution, conjugate, and substrate solutions are ready to use.

TEST PROCEDURE

A. Set-Up of the Assay

Remove the individual kit components and allow them to warm to room temperature (20-25°C). Determine the total number of samples and controls to be tested. The positive calibrator should be run in triplicate. Also, a positive control, negative control, and a reagent blank must be included each time the assay is run. Controls and samples should be run in duplicate until the laboratory becomes proficient with the test procedure. Each sample, calibrator, control, and reagent blank requires one antigen coated microwell.

Determine the number of microwells needed. After the strips and holder have warmed to room temperature cut open the protective envelope and remove the plate containing the antigen coated microwell strips. Strips that are not needed for the assay should be placed into the re-sealable pouch, sealed and returned to storage at 2-8° C.

B. Serum Incubation

Prepare a 1:21 dilution of the positive and negative controls, the positive calibrator and each patient serum as follows:

1. Add 10 μ L of each sample to a separate well of the dilution plate provided. Add 200 μ L of sample diluent to each well containing a sample.
2. Using a multichannel pipette, transfer 100 μ L of each diluted sample and control from the dilution plate to the test plate. Withdraw and expel the samples several times before the final transfer to ensure that the samples are properly mixed. Use a different pipette tip for each sample. Add 100 μ L of sample diluent to a well as a reagent blank.
3. Cover the wells with a plate sealer and incubate the plate at room temperature (20-25°C) for 20 to 22 minutes.
4. Wash the microwell strips 5X.
 - a. Vigorously shake out the liquid from the wells.
 - b. Fill each well with wash buffer. Make sure no air bubbles are trapped in the wells.
 - c. Repeat steps **a.** and **b.** for a total of five washes.
 - d. Shake out the wash solution from all the wells. Invert the plate over a paper towel and tap firmly to remove any residual wash solution from the wells. Visually inspect the plate to ensure that no residual wash solution remains. Collect wash solution in a disposable basin and treat with 0.5% sodium hypochlorite (10% household bleach) at the end of the days run.

NOTE: Autowash - If using an automated wash system, set the dispensing volume to 300-350 μ L/well. Set the wash cycle for 5 washes with no delay between washes. Remove microtiter plate from washer, invert plate over paper towel and tap firmly to remove any residual wash solution from the wells.

C. Conjugate Incubation

1. Add 100 μ L of the conjugate solution to each well at the same rate and in the same order as the specimens were added.
2. Cover the plate with the plate sealer provided and incubate at room temperature (20-25°C) for 20 to 22 minutes.
3. Wash the plate by following the procedure in Step **B.4., a.** through **d.**

D. Substrate Incubation

1. Add 100 μ L of the TMB substrate solution to each well at the same rate and in the same order as the conjugate was added. (One mL of TMB substrate is sufficient for 8 wells).
2. Incubate the plate at room temperature (20-25°C) for 10 to 12 minutes.
3. Add 50 μ L of stop solution to each well at the same rate and the same order as the TMB solution was added. Positive samples will turn from blue to yellow. After adding stop solution, tap plate several times to ensure that the samples are thoroughly mixed.
4. Set the microplate reader to read at a wavelength of 450nm and measure the optical density (OD) of each well against the reagent blank. The plate should be read within 30 minutes after the addition of the stop solution.

QUALITY CONTROL

1. Each time the assay is run, the positive calibrator should be run in triplicate. A positive and negative control, and reagent blank must also be included in each assay.

2. Calculate the mean of the three positive calibrator determinations. If any of the three positive calibrator values differ by more than 15% from the mean, discard that value and calculate the mean of the remaining two values.
3. The mean OD value for the positive calibrator and the OD values for the positive and negative controls should fall within the following ranges:

	<u>OD RANGE</u>
Negative Control	≤ 0.25
Positive Calibrator	≥ 0.30
Positive Control	≥ 0.50

- a. The OD of the negative control divided by the mean OD of the positive calibrator should be ≤ 0.9 .
 - b. The OD of the positive control divided by the mean OD of the positive calibrator should be ≥ 1.25 .
 - c. If the control values are not within the above ranges, the test should be considered invalid and the test should be repeated.
4. The positive control is intended to monitor for substantial reagent failure and will not ensure precision at the assay cut-off.
 5. The positive and negative controls must meet the following additional criteria:
 - a. The negative control must be <12 U/mL
 - b. The positive control must be >25 U/mL
 6. Additional controls may be tested according to guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.

INTERPRETATION OF RESULTS

A. Calculations

1. Positive Calibrator

Based upon testing of normal and disease-state specimens, a maximum normal unit value has been determined by the manufacturer and correlated to the positive calibrator. The calibrator will allow you to determine the unit value of test samples, and to correct for slight day-to-day variations in test results. The calibrator unit value is determined for each lot of kit components and is printed on the vial label.

2. Conversion of Optical Density to U/mL

The conversion of OD to unit value (U/mL) can be represented by the following equation:

$$\text{Test Specimen U/mL} = (A \times B)/C$$

Where: U/mL = Unknown unit value to be determined

A = OD of test specimen in question.

B = Unit value of calibrator (U/mL).

C = The mean OD of calibrator.

Example: Test specimen OD for Cardioliipin = 0.946

Calibrator OD for Cardioliipin = 0.435

Calibrator unit value for Cardioliipin = 155 U/mL

$$\text{Test Specimen U/mL} = (0.946 \times 155)/0.435$$

$$\text{Test Specimen} = 337 \text{ U/mL for anti-Cardioliipin}$$

B. Interpretation of Results

Patient samples may be graded as normal, low positive, moderate, or high positive according to the following recommendations:

	<u>U/mL</u>
Normal	< 12
Equivocal	12-15
Positive	>15

LIMITATION OF THE ASSAY

1. A diagnosis should not be made on the basis of anti-Cardiolipin ELISA results alone. Test results for anti-Cardiolipin should be interpreted in conjunction with the clinical evaluation and the results of other diagnostic procedures.
2. The performance characteristics of this device have not been established for lipemic, hemolyzed and icteric specimens; therefore, these specimens should not be tested with this assay.
3. Although aCL has been associated with certain SLE subsets (1-3), the clinical significance of aCL in SLE and other diseases remains under investigation.
4. The range of "normal" aCL values may vary from population to population. The normal ranges shown above are those recommended by one group of investigators and are supported by studies of random blood donors from Northeastern United States. Testing laboratories, however, are encouraged to establish normal ranges for their regions.
5. The clinical significance of any test result depends upon its relationship to other medical patient data. Disease diagnosis and management should be based on an evaluation of all relevant patient information.

EXPECTED RESULTS

In a study conducted by Diagnostic Automation Inc., 113 normal donor sera from Northeastern United States were evaluated for Cardiolipin IgA autoantibodies. Of the 113 tested, 1/113 (0.9%) was positive, 1/113 (0.9%) was equivocal, and the remainder 111/113 (98%) were negative for anti-Cardiolipin IgA antibody.

In the same study a group of 28 uncharacterized SLE specimens were evaluated for Cardiolipin IgA autoantibodies. Of these 28 specimens, 16/28 (57%) were positive, 3/28 (11%) were equivocal, and 9/28 (32%) were negative.

PERFORMANCE CHARACTERISTICS

Comparative Study:

An in-house comparative study was performed to demonstrate the equivalence of the Diagnostic Automation Inc. Cardiolipin IgA ELISA test system to another commercially

available Cardiolipin IgA ELISA test system. Performance was evaluated using 260 specimens as described in Table 1 below. The results of the investigation have been summarized in Table 2 below.

Table 1. Summary of Clinical Specimens

No.	Comments
105	Disease state specimens obtained from rheumatology groups from two different university hospitals.
14	Specimens previously tested and found positive for anti-Cardiolipin.
28	Uncharacterized SLE patient samples.
113	Normal donor samples collected in Northeastern United States.

Table 2: Calculation of Relative Sensitivity, Specificity, and Agreement

Commercial Cardiolipin IgA ELISA Result

		+	-	Equivocal*	Totals
DAI Cardiolipin IgA ELISA Test System	+	34	46	7	87
	-	2	154	1	157
	Equivocal*	0	16	0	16
	Totals	36	216	8	260

* Equivocal results excluded from all calculations.

Relative Sensitivity = $34/36 = 94.4\%$

95% Confidence Interval** = 87.0 to 100%

Relative Specificity = $154/200 = 77.0\%$

95% Confidence Interval** = 71.2 to 82.8%

Relative Agreement = $188/236 = 80.0\%$

95% Confidence Interval** = 74.5 to 85.0%

**95% confidence intervals calculated using the exact method.

Reproducibility:

To evaluate both intra-assay and inter-assay reproducibility, six specimens were tested; eight replicates each, on each of three days. These results were then used to calculate mean unit values, standard deviations, and percent CV. Two of the specimens were strong positives, two were clearly negative, and two were near the assay cut off. The results of the study have been summarized below.

**Table 3. Diagnostic Automation Inc. Cardiolipin IgA ELISA;
Results of Precision Testing**

Day 1: Intra-Assay Reproducibility.

Specimen	Mean Result (U/mL)	Standard Deviation	Percent (%) CV	Replicates
1	302	18.3	6.1	8
2	154	13.9	9.0	8
3	82	6.3	7.6	8
4	59	5.5	9.2	8
5	4	0.3	8.1	8
6	5	0.5	8.7	8

Day 2: Intra-Assay Reproducibility.

Specimen	Mean Result (U/mL)	Standard Deviation	Percent (%) CV	Replicates
1	338	22.6	6.7	8
2	192	8.1	4.2	8
3	88	5.6	6.4	8
4	62	7.1	11.5	8
5	6	3.2	50.8	8
6	8	0.9	12.0	8

Day 3: Intra-Assay Reproducibility.

Specimen	Mean Result (U/mL)	Standard Deviation	Percent (%) CV	Replicates
1	266	11.7	4.4	8
2	158	12.4	7.8	8
3	84	5.9	7.0	8
4	55	5.6	10.2	8
5	3	0.8	28.9	8
6	5	2.2	43.5	8

Inter-Assay Reproducibility; All Days Combined:

Specimen	Mean Result (U/mL)	Standard Deviation	Percent (%) CV	Replicates
1	302	34.6	11.5	24
2	168	20.6	12.2	24
3	85	6.1	7.2	24
4	59	6.6	11.2	24
5	4	2.4	56.2	24
6	6	1.8	29.8	24

Cross Reactivity:

To investigate the potential for positive reactions due to cross reactive antibodies, fourteen specimens which were reactive for various auto-antibodies were tested on the anti-Cardiolipin test system. Ten of the fourteen (10/14, 71.4%) were negative for anti-Cardiolipin IgA activity, while four of the fourteen (4/14, 28.6%) were positive. The results of this study indicate that the potential for a high degree of cross reactivity with such autoantibodies is not likely.

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