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For Individual Laboratory to Complete:

**Anti-Cardiolipin
Screen
Enzyme Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
Supercedes		

Method: Diamedix Corp., Immunosimplicity®

Manual or in conjunction with one of the Diamedix Automated EIA Systems such as the MAGO Plus, the DSX, or the DS2. For *In Vitro* Diagnostic Use.

Clinical Significance

Anti-phospholipid antibodies are autoantibodies that react with most negatively-charged phospholipids including cardiolipin. Autoantibodies directed against phospholipids, and anti-cardiolipin in particular, have been associated with recurrent venous and arterial thrombosis, thrombocytopenia and spontaneous abortions. The term 'anti-phospholipid syndrome' is used to describe patients with these clinical manifestations. Autoantibodies to cardiolipin are described in many autoimmune diseases. They are frequently found in patients with SLE, in patients with other autoimmune diseases as well as in some individuals with no apparent underlying disease. For pregnant patients, in addition to spontaneous abortions, anti-cardiolipin antibodies have been associated with pregnancy-induced hypertension, pre-eclampsia, and gestational diabetes. Anti-cardiolipin anti-bodies have also been detected in some non-thrombotic neurological disorders like cerebrovascular insufficiency, cerebral ischemia or chorea and in myocardial infarction (1,2,3,4,5).

Anti-cardiolipin antibodies are found in the immunoglobulin classes IgG, IgM and/or IgA. Anti-cardiolipin IgG antibodies show a good correlation to the clinical status of the patient in thrombosis, thrombocytopenia, fetal loss and some neurological disorders. The determination of IgM antibodies may be a valuable indicator in the diagnosis of early autoimmune diseases, whereas IgG antibodies will be found in progressive stages of manifested autoimmune disorders. Anti-IgA antibodies are often associated with IgG antibodies. Anti-cardiolipin IgA antibody levels have also been found to be significantly higher in SLE patients with vascular complications than those without and correlated with a predisposition to thrombosis, thrombocytopenia and fetal loss (6,7,8,9).

The Diamedix anti-Cardiolipin Screen Test Kit is an enzyme immunoassay intended to measure IgG, IgM and IgA antibodies to cardiolipin in human serum. The assay system uses controls that are traceable to the reference sera from E. N. Harris (10). The test can be performed manually or in conjunction with one of the Diamedix Automated EIA Systems. The total assay time is less than 2 hours and results are reported in Units (U) per ml and represent the sum total of IgG, IgM and IgA antibodies present in the patient sample.

Principle of the Procedure

Highly purified bovine cardiolipin is initially bound to microwells and then saturated with highly purified human β_2 -glycoprotein I which is known as a cofactor for the binding of anti-cardiolipin antibodies (11). This coating procedure guarantees reproducible results independent of endogenous β_2 -glycoprotein I. Diluted patient sera and Controls are placed in the microwells and incubated. Anti-cardiolipin antibodies, if present, will bind to the antigen forming antigen-antibody complexes. Residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG, IgM and IgA) is added and will bind to these complexes. Unbound conjugate is removed by aspirating and washing. Substrate is then added and incubated. In the presence of bound enzyme, the substrate is converted to a colored end product. Stop solution is added and the absorbance of this end product is then read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG/IgM/IgA antibodies to cardiolipin present in the sample.

Specimen Collection

Whole blood should be collected by accepted medical techniques. Separated serum should remain at 22°C for no longer than 8 hours. If assays are not completed within 8 hours, serum should be refrigerated (2-8°C). If assays are not completed within 48 hours, or the separated sample is to be stored beyond 48 hours, samples should be frozen at -20°C. Avoid multiple freeze-thaw cycles. Prior to testing, bring frozen sera to room temperature slowly and mix gently, avoiding foam formation. Specimens containing visible particulate matter should be clarified by centrifugation before testing. Grossly contaminated, hemolyzed, lipemic, or icteric specimens should not be used. The NCCLS provides recommendations for collecting and storing blood specimens (12).

CAUTION: Serum samples must not be heat-inactivated prior to use.

Reagents

Each anti-Cardiolipin Screen Test Kit contains reagents for 96 tests.

Antigen Wells Twelve, 8-well microwell breakapart strips, color-coded blue, coated with purified cardiolipin and saturated with human β_2 glycoprotein I.

Negative Control	One vial with black cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned U/ml value is printed on the label.
Cut-Off Control	One vial with blue cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix containing IgG, IgM and IgA antibodies. The assigned U/ml value is printed on the label.
Positive Control	One vial with white cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix containing IgG, IgM and IgA antibodies. The assigned U/ml value is printed on the label.
High Positive Control	One vial with red cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix containing IgG, IgM and IgA antibodies. The assigned U/ml value is printed on the label.
Sample F Diluent	One bottle with blue cap containing 60 ml phosphate buffer with protein stabilizers. Color-coded blue.
Wash X Concentrate (50X)	Two bottles with clear caps containing 20 ml. Each bottle is sufficient to make 1 liter of wash solution.
Conjugate	One bottle with red cap containing 25 ml rabbit anti-human IgG, IgM and IgA, labeled with horseradish peroxidase, diluted in a PBS buffer with protein stabilizers. Color-coded pink.
Substrate H	One amber bottle with brown cap containing 25 ml buffered TMB solution (3,3',5,5' tetramethylbenzidine). The substrate solution may develop a slight blue color upon storage.
Stop P Solution	One bottle with white cap containing 30 ml 1M Hydrochloric acid. CAUTION: Solution is corrosive. Avoid contact with skin or eyes. If contact is made, flush area with copious amounts of water.

Store these reagents at 2 to 8° C.

Other Materials Required

Manual Users:

1. Wash bottle or automated microplate washer
2. Pipettors capable of dispensing appropriate volumes
3. Timer
4. One liter graduated cylinder
5. One liter wash solution reservoir

6. Deionized or distilled water
7. Absorbent toweling
8. Tubes or microwell plate for sample dilution
9. Reader capable of reading absorbance at 450nm, reference at 600-630 nm (Performance characteristics have not been established for a single wavelength reader.)

Diamedix Automated EIA System Users:

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers as appropriate to system
4. Sample and Reagent tips required by system
5. Reagent containers required by system

Warnings and Precautions:

REAGENTS: For in vitro Diagnostic Use

1. Handle samples, standards, controls and the materials that contact them as potential biohazards. Each donor unit in the Calibrator and controls has been found negative for Hepatitis B surface antigen and HIV-1 and -2 antibodies by FDA-approved third generation tests. However, because no method can offer complete assurance that HIV-1 and -2, Hepatitis B virus, or Hepatitis C virus, or other infectious agents are absent, these materials should be handled at the Biosafety Level 2 as recommended for any potentially infectious serum or blood specimen in the Centers for Disease Control/National Institutes of Health Manual, "Biosafety in Microbiological and Biomedical Laboratories", 1993.
2. Never pipette by mouth.
3. Avoid contact with open skin and mucous membranes.
4. Certain components contain sodium azide as preservative. Azides are reported to react with lead and copper in plumbing to form compounds that may become explosive. When disposing of solutions containing sodium azide, flush with copious amounts of water to minimize the build up of metal azide compounds.
5. Sodium azide inhibits horseradish peroxidase activity. Care must be taken to ensure that azide is not carried over from other reagents into conjugate and substrate steps.
6. Avoid contamination of the TMB substrate solution with conjugate or other oxidants which will cause the solution to change color prematurely.
7. The substrate contains 3,3', 5,5' Tetramethylbenzidine (TMB) which has shown possible mutagenic effects in laboratory experiments.

Calibration

This test uses Controls that are traceable to the E.N.Harris reference sera (10). The High Positive, Positive Control, Cut-Off and Negative Control sera have been assigned values of 90 U/ml, 30 U/ml, 10 U/ml and 3.3 U/ml respectively. Semi-quantitative results may be obtained from the point to point or 4-parameter logistic curve fit using all four controls. Samples

with values ≥ 15 U/ml are considered positive for IgG, IgM or IgA antibodies to cardiolipin and samples whose values ≤ 12 U/ml are considered negative for IgG, IgM or IgA antibodies to cardiolipin. To account for the inherent variations in enzyme immunoassays an equivocal range of from 12 to 14.9 U/ml has been included just below the assay cut-off.

Quality Control

- (a) All Controls must be included in each test run.
- (b) The absorbance of the Blank must be < 0.250 .
- (c) The absorbance of the Positive Control must be greater than the absorbance of the Cut-Off Control.
- (d) The absorbance of the High Positive Control must be greater than
3.5 times the
absorbance of the Cut-Off Control.

If any of these criteria is not met, the results are invalid and the test should be repeated.

Note: Additional controls may be tested according to guidelines or requirements of local, state, or federal regulations or accrediting organizations. For guidance on appropriate Quality Control practices please refer to NCCLS C24-A, Internal Quality Control Testing: Principles and Definitions.

Procedure

Allow all test components and patient samples to warm to room temperature before use. Invert reagent bottles gently several times before use. Return promptly to the refrigerator after use.

Prepare Wash Solution by adding 20 ml of Wash Concentrate (50X) to one liter with deionized or distilled H₂O.

Manual Users:

The Controls are provided ready to use: **DO NOT DILUTE FURTHER.**

1. Prepare 1:101 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 5 μ l sample to 500 μ l Sample Diluent).
2. Mix sample dilutions gently by withdrawing and expelling in a pipette tip 2 or 3 times or by vortex mixing for 2 or 3 seconds. Transfer 100 μ l of controls and diluted patient samples, to the antigen wells. Avoid formation of bubbles when transferring diluted samples.
3. Allow the wells to incubate at room temperature (18-30°C) for 30 \pm 5 minutes.
4. Aspirate or discard the contents of the wells. Remove excess moisture in the wells by tapping on paper toweling. Wash the wells by rinsing 3 times with at least 300 μ l of Wash Solution. Remove

- excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
5. Place 100 μ l of Conjugate into each well, avoiding bubble formation.
 6. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 \pm 5 minutes.
 7. Wash the wells as described in Step 4 above.
 8. Place 100 μ l of Substrate into each well, avoiding bubble formation.
 9. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 \pm 5 minutes.
 10. Place 100 μ l of Stop Solution into each well, avoiding bubble formation.
 11. Mix well contents thoroughly.
 12. Read the absorbance of each well at 450 nm. A suitable reference wavelength (e.g., 600-630 nm) should be used.

NOTE: The developed color is stable for 30 minutes. Read the absorbances during this time.

Diamedix Automated EIA System Users:

When using one of Diamedix's Automated EIA Systems, refer to the corresponding Operating Manual for the test setup, procedure, and accessories/consumables needed.

Calculation of Results

Semi-quantitative results may be obtained from the point to point or 4-parameter logistic curve fit using all four Controls. For the BP-96 or Stat-Fax readers the point-to-point option should be selected and Control values entered accordingly. The Diamedix Automated EIA Systems will calculate and print results automatically.

Reference Ranges

The following is only a guide to interpretation. **Each laboratory can establish its own "normal" ranges based on populations encountered.**

U/ml Value	
< 12.0	Negative, No detectable IgG, IgM or IgA antibodies to cardiolipin.
\geq 15.0	Positive IgG, IgM or IgA antibody to cardiolipin detected.
12.0-14.9	**Equivocal for IgG, IgM or IgA antibodies to cardiolipin.

** Equivocal samples can be retested by this method, tested by another method or a new sample tested. Alternatively, equivocal samples can be tested on the specific IgG, IgM or IgA anti-Cardiolipin kits.

Further confirmation of positive results should be carried out by testing samples using the specific IgG, IgM or IgA anti-Cardiolipin Test Kits.

NOTE: The anti-Cardiolipin Screen Test Kit recognizes the sum of IgG, IgM and IgA-class antibodies. Due to additive effects, patient samples giving low positive results in the anti-Cardiolipin Screen may be determined as negative using the specific anti-IgG, IgM or IgA assay.

Procedure Notes

1. Do not interchange reagents from different reagent lots except for Sample **F** Diluent, Wash **X** Concentrate, Substrate **H** and Stop **P** Solution.
2. Do not use reagents beyond their expiration date.
3. Store unused reagents at 2 to 8°C.
4. Incubations above or below the recommended temperatures or times may give erroneous results.
5. The EIA method is a very sensitive technique. Maintain consistent pipetting technique, incubation times and temperature conditions throughout the test procedure. Cross contamination between reagents can invalidate the test.
6. Coated microwells should be stored with the desiccant in the resealable bag provided and returned to the refrigerator immediately after use.
7. *(Manual Procedure Only)* The washing procedure is very important and requires special attention. (Please refer to the Procedure section)
NOTE: *Improperly washed wells may give erroneous results.*
8. The concentration of anti-Cardiolipin antibodies in a given specimen determined with assays from different manufacturers can vary due to differences in assay methods and reagent specificity.

Limitations

1. The results obtained with the Is-anti-Cardiolipin Screen Test Kit serve only as an aid to diagnosis and should not be interpreted as diagnostic in themselves. Results must be interpreted in conjunction with the patient history, clinical symptoms, physical findings as well as other diagnostic procedures.
2. The clinical significance of elevated anti-cardiolipin antibody levels in diseases other than SLE is still under investigation.
3. When a normal anti-cardiolipin antibody level is found in the presence of clinical manifestations, a lupus anti-coagulant or other additional testing is indicated.

4. Treatment should not be initiated on the basis of a positive anti-cardiolipin level alone. Supportive clinical indications must also be present.
5. In published studies the prevalence of anti-cardiolipin antibodies in SLE generally ranges from approximately 20% to 60%.
6. Assay performance characteristics have not been established for visual result determination or for spectrophotometry using a single wavelength.
7. The test should be performed on serum. The use of whole blood or plasma has not been established.
8. The performance characteristics of the *Is-Anti-Cardiolipin Screen Test Kit* with automated equipment other than one of the Diamedix Automated EIA Systems have not been established.

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