

**For Individual Laboratory to Complete:**

**Anti-Cardiolipin  
IgG/IgM  
Enzyme Immunoassay**

Laboratory Name		
Adopted		
Reviewed		
Reviewed		
Revised		
Supercedes		

**Method:** Diamedix Corp., **Immunosimplicity<sup>®</sup>**

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 IgG/IgM Test Kit is an enzyme immunoassay intended to measure IgG and/or IgM antibodies to cardiolipin in human serum. 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 GPL or MPL Units per ml which are traceable to the reference sera from E. N. Harris (10).

### **Principle of the Procedure**

Highly purified bovine cardiolipin is initially bound to microwells and then saturated with highly purified human  $\beta_2$  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  $\beta_2$  glycoprotein I. Diluted patient sera, Standards 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 or IgM) 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 or IgM 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 Is-anti-Cardiolipin IgG/IgM 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  $\beta_2$  glycoprotein I.

Standard A(0 GPL/0 MPL)

One vial with yellow cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.

Standard B (7.5 GPL/5 MPL)	One vial with green cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Standard C (15 GPL/10 MPL)	One vial with brown cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Standard D (30 GPL/20 MPL)	One vial with purple cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Standard E (60 GPL/40 MPL)	One vial with white cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Standard F (120 GPL/80 MPL)	One vial with red cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix. The assigned value is printed on the label.
Negative Control	One vial with black cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix, negative for cardiolipin IgG and IgM antibodies. The assigned range is printed on the label.
Positive Control	One vial with blue cap containing 1.8 ml of pre-diluted human serum or defibrinated plasma in a PBS/BSA matrix, moderately reactive for IgG and IgM antibodies. The assigned range is printed on the label.
Sample <b>F</b> Diluent	One bottle with blue cap containing 60 ml phosphate buffer with protein stabilizers. Color-coded blue.
Wash <b>X</b> Concentrate	Two bottles with clear caps containing 20 ml. Each (50X) bottle is sufficient to make 1 liter of wash solution.
IgG Conjugate	One bottle with red cap containing 25 ml rabbit anti-human IgG labeled with horseradish peroxidase, diluted in a PBS/BSA matrix. Color-coded pink.
IgM Conjugate	One bottle with red cap containing 25 ml rabbit anti-human IgM labeled with horseradish

peroxidase, diluted in a PBS/BSA matrix.  
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

### **Diamedix Automated EIA System Users:**

1. One liter graduated container
2. Deionized or distilled water
3. Dilution containers 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 immunocomplex 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 Standards that are traceable to the E.N. Harris reference sera (10). The Highest Standard (Std F) has been assigned a value of 120 GPL/80 MPL/ml. The other Standards have been assigned values of: Std E(60 GPL/40 MPL), Std D(30 GPL/20 MPL), Std C(15 GPL/10 MPL), Std B(7.5 GPL/5 MPL), Std A(0 GPL/0 MPL) respectively. Semi-quantitative results may be obtained from the point to point curve fit or 4-parameter logistic curve fit using all six Standards or from the point to point curve fit using three Standards (A,C and F). Samples with values  $\geq 12$  GPL or MPL U/ml are considered positive for IgG or IgM antibodies to cardiolipin and samples whose values  $< 10$  GPL or MPL U/ml are considered negative for IgG or IgM antibodies to cardiolipin. To account for the inherent variations in enzyme immunoassays an equivocal range of from 10 to 11.9 GPL or MPL U/ml has been included just below the assay cut-off.

## **Quality Control**

- (a) The Positive and Negative Controls must be included in each test run and must be within their assigned ranges.
- (b) The absorbance of Standard A(0 GPL/0 MPL) must be  $< 0.200$ .
- (c) The absorbance of Standard F(120 GPL/80 MPL) must be greater than 3 times the absorbance of Standard C(15 GPL/10 MPL).
- (d) The absorbance of Standard C(15 GPL/10 MPL) must be greater than the absorbance of Standard B(7.5 GPL/5 MPL).
- (e) The absorbance of Standard D(30 GPL/20 MPL) must be greater than the absorbance of Standard C(15 GPL/10 MPL).

(f) The absorbance of Standard E(60 GPL/40 MPL) must be greater than the absorbance of Standard D(30 GPL/20 MPL).

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

(For 3-point calibration, d, e and f do not apply).

**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 H<sub>2</sub>O.

### **Manual Users:**

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

***The assays can be performed either using all six Standards and a 6-point Calibration system or by using three Standards, namely Stds. A, C and F, and a 3-point Calibration system. Positive and Negative Controls must be run for either assay option.***

1. Prepare 1:101 dilutions of the patient samples in Sample Diluent. (e.g., by addition of 5 µl sample to 500 µl 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 µl of Standards (three or six), 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 ± 5 minutes.
4. Aspirate or discard the contents of the wells. Remove excess moisture in the wells by tapping on paper toweling if necessary. Wash the wells by rinsing 3 times with at least 300 µl of Wash Solution. Remove excess moisture from the wells after washing. When using an automated washer, follow the manufacturer's instructions.
5. Place 100 µl of Conjugate into each well, avoiding bubble formation.

6. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.
7. Wash the wells as described in Step 4 above.
8. Place 100 µl of Substrate into each well, avoiding bubble formation.
9. Allow the wells to incubate uncovered at room temperature (18-30°C) for 30 ± 5 minutes.
10. Place 100 µl 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 of 600-630 nm reading 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, please 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 curve fit or 4-parameter logistic curve fit using all six Standards or from the point to point curve fit using three Standards (A,C and F). The Diamedix Automated EIA Systems will calculate and print results automatically for either assay option.

**Reference Ranges**

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

**GPL or MPL Value**

- |              |   |
|--------------|---|
| < 10.0       | Negative, No detectable IgG or IgM antibodies to cardiolipin. |
| ≥ 12.0       | Positive IgG or IgM antibody to cardiolipin detected.         |
| 10.0 to 11.9 | **Equivocal for IgG or IgM antibodies to cardiolipin.         |

\*\* Equivocal samples can be retested by this method, tested by another method or a new sample tested.

Samples which yield absorbances greater than that of Standard F (120 GPL/80 MPL) may be reported as 'greater than 120 GPL/80 MPL'. Alternatively, such

samples may be pre-diluted in Sample Diluent and retested. The resulting GPL/MPL U/ml value must be multiplied by the dilution factor for reporting.

*Example: If the specimen was pre-diluted 1:5 before testing,  
The resulting GPL/MPL U/ml should be multiplied by 5.*

## **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 concentrations of anti-cardiolipin IgG and/or IgM 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 IgG/IgM 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. In published studies the prevalence of anti-cardiolipin antibodies in SLE generally ranges from approximately 20% to 60%.
5. Assay performance characteristics have not been established for visual result determination or for spectrophotometry using a single wavelength.
6. The test should be performed on serum. The use of whole blood or plasma has not been established.
7. High levels of bilirubin, hemoglobin, icteric substances, protein and immunoglobulins in samples may affect the results and thus should be avoided.



8. Performance characteristics of the Is anti-Cardiolipin IgG/IgM Test Kit with automated equipment other than one of the Diamedix Automated EIA Systems have not been established.

## References

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