

**CARDIOLIPIN ANTIBODY (IgM) TEST**

Product No. 3100

**IVD** For *In Vitro* Diagnostic Use

**INTENDED USE**

The ImmunoWELL Cardiolipin Antibody (IgM) Test is an enzyme immunoassay (EIA) for the quantitative detection of antibodies to cardiolipin antigen in serum and is used as an aid for assessing the risk of thrombosis in individuals with systemic lupus erythematosus (SLE) and lupus-like disorders.

**SUMMARY AND EXPLANATION**

Anticardiolipin (aCL) autoimmune antibodies are a subset of antibodies which react with negatively charged phospholipids. One type of anti-phospholipid, lupus anticoagulant (LA), is frequently found in systemic lupus erythematosus (SLE) patients. LA is defined as an immunoglobulin that prolongs *in vitro* coagulation tests (e.g., APTT or PT). Anticardiolipin antibodies are also frequently observed in patients with SLE. One autoantibody, LA, is measured with a bioassay and the other autoantibody, aCL, is measured based on affinity for cardiolipin. In many cases both autoantibodies are present in a single patient and may be the same antibody in some cases. Thrombosis and spontaneous abortion are among the symptoms common to SLE patients; in fact, elevated levels of anticardiolipin antibodies have been strongly associated with the presence of both venous and arterial thrombosis, thrombocytopenia, and recurrent fetal loss.<sup>1,2</sup> Patients who present with these manifestations are known to have an "anti-phospholipid syndrome".<sup>2,3,4,5,6</sup>

Anticardiolipin (aCL) and lupus anticoagulant (LA) are not limited to SLE patients. In fact, the majority of people who have these autoantibodies do not have SLE. These antibodies occur in patients with other autoimmune diseases and in individuals with no apparent autoimmune disease<sup>3,7,8</sup>; yet, many subjects manifest one or more of the clinical complications associated with the presence of anti-phospholipid antibodies in their circulation.

The ImmunoWELL Cardiolipin Antibody (IgM) Test is an EIA which measures IgM aCL in human serum. The ImmunoWELL test provides highly reproducible results expressed in units that are standardized against an internationally recognized reference preparation. Levels of IgM aCL antibodies are reported as MPL units. One MPL unit is defined as the cardiolipin binding activity of 1 µg/ml of an affinity purified IgM aCL preparation from a standard serum (DUF)<sup>9</sup>.

**ASSAY PRINCIPLE**

The ImmunoWELL Test utilizes an EIA microtiter plate technique for the detection of antibodies. Serum is added to antigen coated microtiter wells and allowed to react. After removal of unbound antibodies, horseradish peroxidase-conjugated antihuman antibodies are allowed to react with bound antibodies. The bound peroxidase reacts with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS®), the chromogenic substrate, developing a color. Finally, the substrate reaction is stopped and the optical density is read with a spectrophotometric microwell reader.

**REAGENTS**

**Reaction Wells** coated with diphosphatidyl glycerol (cardiolipin from bovine heart).

**Specimen Diluent:** Consisting of 0.01M phosphate buffered saline (PBS, pH 6.2-7.6) and 10% newborn calf serum containing <0.1% NaN<sub>3</sub>.

**Positive Control:** Consisting of human anti-cardiolipin serum containing <0.1% NaN<sub>3</sub>.

**Negative Control:** Consisting of nonreactive human serum containing <0.1% NaN<sub>3</sub>.

**Calibrators (3):** Consisting of human anti-cardiolipin sera at specified concentrations containing <0.1% NaN<sub>3</sub>.

**Wash Buffer Concentrate:** Consisting of a 20X concentrate of 0.01M PBS (pH 6.2-7.6)

**Conjugate:** Consisting of peroxidase-conjugated goat antihuman IgM in PBS (pH 6.2-7.6) and carrier protein containing preservative

**Substrate Buffer** consisting of 0.1 M sodium citrate (pH 4.4-4.6) and 0.01% hydrogen peroxide

**Substrate Concentrate** 2.19% 2-2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) in 0.1 M sodium citrate (pH 4.4-4.6)

**Stop Solution** 0.25 M Oxalic Acid

**Warnings and Precautions**

**For *In Vitro* Diagnostic Use:** ImmunoWELL reagents have been optimized for use as a system. Do not substitute other manufacturers' reagents or other ImmunoWELL Test reagents. Dilution or adulteration of these reagents may also affect the performance of the test. Do not use any kits beyond the stated expiration date. Close adherence to the test procedure will assure optimal performance. Do not shorten or lengthen stated incubation times since this may result in poor assay performance.

Some reagents contain sodium azide that may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build-up.

**Warning - Potential Biohazardous Material:** Human sera used in the preparation of this product were tested using FDA approved procedures and found non-reactive for hepatitis B surface antigen and for antibodies to HIV-1, HIV-2, and hepatitis C virus. Because no test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting disease<sup>10</sup>.

**Reconstitution and Storage**

**Kit** is stored at 2-8°C until stated expiration date.

**Reaction wells** are removed from the foil pouch and unused wells are resealed in the pouch using the integral zip-lock.

**Wash Buffer** (pH 6.2-7.6) is prepared by adding the contents of the Wash Buffer Concentrate (20X) bottle into 1 liter of distilled/deionized water. After reconstitution, the 1X solution is stored at 2-8°C. Discard when visibly turbid.

Note: In some instances the Wash Buffer Concentrate (20X) may develop crystals upon storage at 2-8°C. It is important that these crystals are completely redissolved before dilution of the Concentrate. This can be accomplished by warming the Concentrate to 37°C in a water bath with occasional mixing.

**Color Developer** is prepared by adding one (1) drop of Substrate Concentrate to 1mL of Substrate Buffer. One mL of Color Developer is sufficient for one eight-well strip. **Use within one hour.**

**SPECIMEN COLLECTION AND HANDLING**

ImmunoWELL Test is performed on serum. The test requires 10 µL of serum. Serum is collected according to standard practices and may be stored at 2-8° C for up to five days. Serum may be frozen below -20° C for extended periods.

**PROCEDURE**

**Materials Provided**

|                             |                               |
|-----------------------------|-------------------------------|
| Microtiter Wells in Carrier | Specimen Diluent              |
| Calibrators                 | Positive Control              |
| Negative Control            | Wash Buffer Concentrate (20X) |
| Conjugate                   | Substrate Buffer              |
| Substrate Concentrate       | Stop Solution                 |
| Package Insert              |                               |

## Materials Required But Not Provided

|                                      |            |
|--------------------------------------|------------|
| Distilled or deionized water         | Pipets     |
| Microwell washer                     | Test tubes |
| Microwell spectrophotometer (405 nm) |            |

## Performance Considerations

Reproducibility in the assay is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the assay procedure.

**Positive and Negative Control Sera (Undiluted)** are used to assure test performance.

**Calibrators (prediluted)** are used to construct a standard curve.

**Substrate Blank** - All reagents, except serum, are added to the substrate blank well. This blank well is intended to baseline (zero) the microwell spectrophotometer.

## Assay Procedure

1. Allow all components including diluted Wash Buffer to warm to room temperature (22-27°C).
2. Determine the total number of specimens to be tested. Include one blank and duplicates of calibrators and controls in each run. Single test points are adequate for specimen testing, but duplicate tests are recommended for the controls and calibrators.
3. For each control and specimen, pipet 10 µL serum into a clean tube containing 1 mL Specimen Diluent and mix (1:100 dilution).

### CAUTION: Calibrators are prediluted. Do not dilute further.

4. Determine the total number of wells to be run including blank, calibrators, controls, and specimens. Well strips can be broken to the exact number needed to conserve reagent wells. Strips need to be completed with used wells to facilitate washing procedures.
5. Add 100 µL of Specimen Diluent to the first well as a substrate blank. Pipet 100 µL of the prediluted calibrators and the diluted controls and specimens into each assigned well.
6. Incubate at room temperature (22-27°C) for 60±2 minutes.
7. Aspirate the samples out of the wells.
8. Wash the wells three times by completely filling the wells with Wash Buffer (see Reconstitution and Storage) and aspirating the wells completely after washes.
9. Pipet 100 µL Conjugate into all wells.
10. Incubate the wells at room temperature (22-27°C) for 30±1 minutes.
11. Aspirate the conjugate out of the wells.
12. Wash the wells three times as described in step 8.
13. Prepare fresh Color Developer (see Reconstitution and Storage).
14. Pipet 100 µL of Color Developer into each well.
15. Incubate at room temperature (22-27°C) for 30±1 minutes.
16. Add 100 µL of Stop Solution to each well.
17. Inspect the outside bottom surface of the microwells for the presence of condensation, dried buffer salts or wash solution, which might interfere with the spectrophotometric reading. Carefully clean the well bottoms with a soft tissue.
18. Using the substrate blank to zero the spectrophotometer, read the optical density of each well at 405 nm within 30 minutes of completion of step 16.

NOTE: It is recommended that dual wavelength spectrophotometers use only one wavelength, 405 nm.

## Quality Control

The average of the Positive Control must be within the expected range. Determination of this range is described in the Supplement to this package insert which is included with this reagent kit. Because test methods may vary between laboratories, it is recommended that the laboratory maintain a record of control values to assure testing consistency and use the expected range as a guideline.

The assigned values of the Positive Control and Calibrators will vary by lot number. Please verify that the lot number on the vial matches the lot

number on the Package Insert Supplement to assure proper values are used in determinations. Calibrator values (GPL units) were assigned by simultaneous testing with the standards of Dr. E.N. Harris of the Anti-phospholipid Standardization Laboratory, University of Louisville<sup>11</sup>.

## INTERPRETATION

Construct a point-to-point standard curve using the absorbance values **you** observe and their corresponding assigned values. Use this curve to calculate antibody concentration of controls and specimens.

Levels of *aCL* representing clinical significance are not well established. It is recommended that reports be made according to the following<sup>11</sup>:

|                   | <u>MPL Units</u> |
|-------------------|------------------|
| High Positive     | >60              |
| Moderate Positive | >20 – 60         |
| Low Positive      | 10 – 20          |
| Negative          | <10              |

## LIMITATIONS

The anti-cardiolipin activity values obtained from the assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures. If clinical findings suggest the presence of anti-phospholipid antibodies, and the patient is negative for anti-cardiolipin antibodies, some investigators recommend testing for the lupus anticoagulant to confirm the negative result. A patient is considered positive for anti-phospholipid antibodies if one or both of the tests give positive results<sup>12</sup>.

Patients with current or prior syphilis infections may have a positive result in the GenBio Cardiolipin Antibody (IgG) Test without risk of thrombosis. If a patient's history is suggestive of syphilis, this possible diagnosis should be ruled out by an assay specific for anti-treponemal antibodies. Anti-cardiolipin antibodies can appear transiently during many infections. If a patient first tests positive while there are clinical signs of infection, the test should be repeated after an interval of 6 months<sup>13</sup>.

## EXPECTED RESULTS

Although the limit of sensitivity to measure the *aCL* analyte is 10 MPL units, the limit (cutoff) which measures a level of antibody significantly above a normal, asymptomatic population is higher. This limit was determined by measuring serum in forty-one normal, asymptomatic subjects. The average titer of *aCL* was 3.6 MPL units with a range between 0 and 11 MPL units and a standard deviation of 3.7 MPL units. These data further support that values above approximately 15 MPL units are significantly different than expected within a normal population.

Thirty-six (36) serum samples from individuals diagnosed as having SLE were tested with the ImmunoWELL Cardiolipin Antibody (IgM) Test. Eleven of the 36 sera were classified as positive based on a 10 MPL cutoff. Four patients showed low antibody levels, five had moderate antibody titers, and two exhibited a high response. Using an interpretation based on the likelihood of difference from results in normal patients, seven of the thirty-six patients had antibody levels above the 15 MPL cutoff (three standard deviations above the mean of normals). Four of these seven patients also had significant elevation of the IgG *aCL*.

## PERFORMANCE CHARACTERISTICS

Twenty-five (25) presumptive positive serum samples were tested using the ImmunoWELL Cardiolipin Antibody (IgM) Test and the results were compared to an EIA test performed by a clinical reference laboratory. Discrepant samples were retested using an alternate, commercial EIA assay. Both the ImmunoWELL test and the reference lab EIA test normalize data against internationally recognized standards (Antiphospholipid Standardization Laboratory) for anticardiolipin antibodies (*aCL*). ImmunoWELL performed essentially the same as the reference test method, showing better than 99% correlation. Eight of the 25 samples were above a cutoff of 15 MPL units in the reference test. Of these, two were high positives in both the reference and ImmunoWELL tests. Five specimens tested as moderate positives in the reference laboratory, but only three of these were positive in the ImmunoWELL assay. The two samples which tested negative (15 and 23 MPL in the reference lab) also tested negative in the alternate, commercial test. ImmunoWELL found one of the presumptive positives which tested

negative in the reference laboratory (13 MPL) to be a low positive (16 MPL).

Forty-one (41) normal, asymptomatic sera collected from blood donors were tested with the ImmunoWELL Cardioliipin Antibody (IgM) Test. Using the ASL guidelines described under the Interpretation Section, 37 (90%) of the normal samples were negative and 4 (10%) were classified as low positive. No sample had an antibody level significantly higher than the mean of these normals (>15 MPL). Using a cutoff level of 15 MPL units, the overall specificity of the ImmunoWELL Cardioliipin Antibody (IgM) Test is >99% (41/41).

To measure reproducibility, a series of samples were tested in triplicate in five different runs. The results are shown in Table 1.

**Table 1: Reproducibility**

| <u>Mean (MPL)</u> | <u>Within Assay Variation</u> | <u>Between Assay Variation</u> |
|-------------------|-------------------------------|--------------------------------|
| 14                | 15.2%                         | 44.0%                          |
| 27                | 5.0%                          | 13.7%                          |
| 59                | 4.0                           | 10.6%                          |
| 70                | 5.5%                          | 18.7%                          |
| 88                | 4.8%                          | 12.5%                          |

Variation is presented as Coefficient of Variation (%CV).

**BIBLIOGRAPHY**

1. Elias, M and A Eldor, 1984. Thromboembolism in patients with the "lupus"-type circulating anticoagulant, *Arch Intern Med* 144: 510-515.
2. Harris, EN, AE Gharavi, ML Boey, BM Patel, CG Mackworth-Young, S Loizou, and GRV Hughes, 1983. Anticardiolipin antibodies: Detection by Radioimmunoassay and Association with Thrombosis in Systemic Lupus Erythematosus. *Lancet ii*: 1211-1214.
3. Buchanan, RRC, JR Wardlaw, AG Riglar, GO Littlejohn, and MH Miller, 1989. Anti-phospholipid antibodies in Connective Tissue Diseases: Their Relation to the Anti-phospholipid Syndrome and forme fruste Disease *J Rheumatol* 16: 757-61.
4. Hughes, GRV, EN Harris, and AE Gharavi, 1986. The Anticardiolipin Syndrome *J Rheumatol* 13(3): 486-489.
5. Asherson, RA and EN Harris, 1986. Anticardiolipin Antibodies-Clinical Associations *Postgraduate Med J* 62: 1081-1087.
6. Asherson, RA, 1989. Antibodies to Phospholipid and the "Anti-phospholipid Syndrome." *Immunology Updates* 1(2): 1-4.
7. Harris, EN, AE Gharavi, and GRV Hughes, 1985. Anti-phospholipid Antibodies. *Clin Rheum Dis* 11(3): 591-609.
8. Fields, RA, H Toubbeh, RP Searles, and AD Bankhurst, 1989. The Prevalence of Anti-cardiolipin Antibodies in a Healthy Elderly Population and its Association with Antinuclear Antibodies. *J Rheumatol.* 16: 623-5.
9. Harris, EN, AE Gharavi, SP Patel and GRV Hughes, 1987. Evaluation of the anti-cardiolipin test: report of an international workshop held 4 April 1986, *Clin Exp Immunol* 68: 215-222.
10. Centers for Disease Control / National Institutes of Health Manual *Biosafety in Microbiological and Biomedical Laboratories* (1983).
11. Package Insert for Calibration Samples. From the Antiphospholipid Standardization Laboratory, University of Louisville. E Nigel Harris, M.D., Director.
12. EN Harris, 1988. Solid Phase Anti-cardiolipin Test Revisited. *Am J Med* 85: 599-601.
13. JE Moore and CF Mohr, 1952. Biologically False Positive Serologic Tests for Syphilis: Type, Incidence, and Cause. *JAMA* 150(5): 467-473.

**QUICK REFERENCE PROCEDURE**

**ImmunoWELL Cardioliipin IgM**

- Prepare Wash Buffer from Wash Concentrate
- Dilute each control and specimen 1:100 in Specimen Diluent
- Add 100 µL of Specimen Diluent into the first well as a substrate blank.
- Pipet 100 µL of the prediluted calibrators and the diluted controls and specimens into coated microwells and incubate 60 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Pipet 100 µL of Conjugate into microwells and incubate 30 min at room temperature.
- Aspirate microwells and wash microwells three times with Wash Buffer.
- Prepare fresh Color Developer
- Pipet 100 µL of Color Developer into microwells and incubate 30 min at room temperature.
- Pipet 100 µL of Stop Solution into microwells and read results at 405 nm.

To place an order for ImmunoWELL products, contact your local distributor or call GenBio directly for the distributor nearest you and for additional product information.

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