

DiaPharma IgA Anti-Cardiolipin Semi-Quantitative Test Kit

For In Vitro Diagnostic Use

An enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative determination of IgA anti-cardiolipin antibodies in human serum or plasma.

INTENDED USE

For the detection and semi-quantitation of anti-cardiolipin antibodies in individuals with systemic lupus erythematosus (SLE) and lupus-like disorders (anti-phospholipid syndrome).

SUMMARY AND EXPLANATION OF THE IgA ANTI-CARDIOLIPIN TEST

Anti-phospholipid antibodies are autoantibodies that react with most negatively charged phospholipids, including cardiolipin (CL).^{1,2} Additionally, anti-phospholipid antibodies are known to prolong *in vitro* phospholipid-dependent coagulation tests and have been historically referred to as the "lupus anticoagulant".^{1,3,4} Paradoxically, patients with the lupus anticoagulant do not present with abnormal bleeding except in the presence of other hemostatic abnormalities.³

Anti-cardiolipin (aCL) antibodies are frequently found in patients with systemic lupus erythematosus (SLE). They are also found in patients with other autoimmune diseases, as well as in some individuals with no apparent previous underlying disease.^{1,5,6} Elevated levels of aCL antibodies have been reported to be significantly associated with the presence of both venous and arterial thrombosis, thrombocytopenia, and recurrent fetal loss. The term "anti-phospholipid syndrome" (APS) has been introduced to describe patients who present these clinical manifestations, in association with aCL antibodies or the lupus anticoagulant.^{6,7,8,9,10}

High serum levels of IgG aCL antibodies are more prevalent and more clinically relevant than IgM aCL antibodies when correlated with clinical manifestations of the APS.^{11,12,13,14} Studies indicate that elevated serum levels of IgA aCL antibodies are also frequently found in patients with SLE and related disorders.^{2,15,16,17} IgA aCL serum levels were significantly higher in SLE patients with vascular complications than those without,¹⁵ and correlated with a predisposition to thrombosis, thrombocytopenia, and fetal loss.^{16,18}

The DiaPharma IgA Anti-Cardiolipin Test Kit uses a well known ELISA format capable of detecting a specific isotype of aCL antibodies in human serum or plasma. Solid-phase immunoassays are generally considered more sensitive¹⁰ and more specific¹⁹ for detecting aCL antibodies than coagulation assays. The DiaPharma IgA Anti-Cardiolipin Test Kit provides rapid, highly reproducible, accurate, and objective results in units that have been standardized against a reference preparation. The values for IgA aCL antibodies are reported in APL (IgA antiphospholipid) units.

PRINCIPLE OF THE TEST

The test is performed as an indirect ELISA. Diluted serum samples, calibrator sera, and controls are incubated in cardiolipin coated microwells, allowing aCL antibodies present in the samples to react with the immobilized antigen. After the removal of unbound serum proteins by washing, antibodies specific for human IgA labeled with horseradish peroxidase (HRP) are added forming complexes with the cardiolipin bound antibodies. Following another washing step, the bound enzyme-antibody conjugate is assayed by the addition of tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) as the chromogenic substrate. Color develops in the wells at an intensity proportional to the serum concentration of IgA aCL antibodies.

Results are obtained by reading the O.D. (optical density or absorbance) of each well with a spectrophotometer. Calibrator serum is provided, with the IgA aCL concentration expressed in APL units. Dividing the concentration value of the calibrator by the O.D. value of the calibrator provides a conversion factor. The O.D. values of the controls and patient samples are multiplied by the conversion factor to obtain IgA aCL values, expressed in APL units.

REAGENTS

Store at 2 - 8°C. Do Not Freeze.

Each DiaPharma IgA Anti-Cardiolipin 96-microwell Test Kit contains the following reagents (**volumes may vary depending on kit size and configuration**):

- 96 stabilized beef heart cardiolipin (diphosphatidyl glycerol) coated microwells (12 strips of 8 breakaway wells), with frame.
- 1 bottle (60 mL) Sample Diluent (green solution); contains bovine calf serum.*
- 1 vial (0.150 mL) aCL IgA Calibrator Serum (human); see vial label for antibody concentration in APL units.*
- 1 vial (0.150 mL) aCL IgA Positive Control Serum (human); see vial label for expected APL range.*
- 1 vial (0.150 mL) aCL Normal Control Serum (human); see vial label for expected APL range.*
- 1 bottle (12 mL) anti-human IgA (goat) HRP-conjugated antibody solution (orange solution); contains 0.01% thimerosal and gentamycin sulfate as preservatives.
- 1 bottle (13 mL) One Component Substrate (TMB and H₂O₂); ready to use.
- 1 bottle (15 mL) Stopping Solution (0.36 N sulfuric acid).
- 1 bottle (30 mL) Wash Concentrate (33X PBS).

*** CAUTION: Contains sodium azide**

WARNINGS AND PRECAUTIONS

For In Vitro Diagnostic Use

1. Human sera used to prepare the calibrator and controls included in this kit have been tested and shown to be negative for antibodies to HBsAg, HCV and HIV I & 2 by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.
2. Do not pipette by mouth.
3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
4. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
5. Certain components of this product contain sodium azide as a preservative. Sodium azide has been reported to form lead and copper azides when left in contact with these metals. These metal azides are explosive. Any solutions containing azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system.
6. One Component Substrate contains a flammable liquid that can cause irritation to the eyes and skin. Absorption through the skin is possible. Use gloves when handling and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.

SPECIMEN COLLECTION AND PREPARATION

Serum is the preferred sample matrix. Blood should be collected by venipuncture, and the serum separated from the cells by centrifugation after clot formation. If not tested immediately, the specimens should be stored at 2 to 8°C. If specimens are to be stored for more than 72 hours, they should be frozen at -20°C or below. Avoid repeated freezing and thawing. Do not use hemolyzed, icteric, or lipemic serum or plasma as these conditions may cause aberrant results. Specimens containing visible particulate matter should be clarified by centrifugation before testing.

Plasma collected with most anticoagulants except heparin may be used if it is tested immediately, or if the platelets have been removed by centrifugation at 1500g for 10 minutes. Blood should be collected by venipuncture and the plasma separated from the cells by centrifugation. The supernatant must be carefully removed after centrifugation to avoid contamination with platelets. Repeating the centrifugation and separation steps may be advisable to minimize platelet contamination. Lysed or aged platelets can react with anti-phospholipid antibodies leading to aberrant results.

INSTRUCTIONS FOR USE

Materials Provided:

DiaPharma IgA Anti-Cardiolipin Test Kit; see "Reagents," page 2 for a complete listing.

Materials Required but not Supplied:

- Reagent grade water to prepare PBS wash solution (1L) and to zero or blank the plate reader during the final assay step.
- Graduated cylinders
- Precision pipettors capable of delivering between 5 µL and 1000 µL, with appropriate tips
- Miscellaneous glassware appropriate for small volume handling
- Flask or bottle, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves
- Plate reading spectrophotometer capable of reading absorbance at 450 nm (with a 650 nm reference if available)
- Multichannel pipettors capable of delivering to 8 wells simultaneously

Procedural Notes

1. Bring serum samples and kit reagents to room temperature and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated storage as soon as possible.
2. All dilutions of calibrator, control, and test sera must be made just prior to use in the assay.
3. A water blank well should be set up on each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL of reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to zero or blank against this water well.
4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution from a plastic squeeze bottle with a wide tip into the bottom of the microwells. No interference is caused if the water blank well receives PBS. An automated microtiter plate washing system can also be used.
5. **IMPORTANT:** Failure to adequately remove residual PBS can cause inconsistent color development of the substrate solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and provides more uniform incubation and reaction times for all wells.

7. Carefully controlled timing of all steps is critical. All calibrators, controls, and samples must be added within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18 - 26°C) may contribute to inaccurate results.
11. Avoid contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use Tween 20 or other detergents in this assay.
13. Do not use kit components beyond expiration date.
14. Do not use kit components from different kit lot numbers.

Reagent Preparation

Wash Solution (PBS): Measure 30 mL of Wash Concentrate (33X PBS) and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1 . Store unused PBS solution in the refrigerator at 2 - 8°C . Discard if the solution shows signs of microbial or cross-contamination.

Assay Procedure

1. Assay the calibrator and controls in duplicate. It is advised that duplicate determinations be made for IgA anti-cardiolipin antibodies in each sample. One well should be run as a reagent blank control; Sample Diluent without serum is added to the well as explained in step 5 of this section. This well will be treated the same as a control or patient sample in subsequent assay steps. A water blank well should be included with each plate; it is to remain empty until 200 μ L of reagent grade water is added at the completion of the assay, immediately prior to reading the plate. The water blank well is to be used to zero the plate reader.
2. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
3. Prepare a 1:50 dilution of the calibrator, controls, and patient samples in Sample Diluent (green solution); e.g., 10 μ L sample added to 490 μ L Sample Diluent equals a 1:50 sample dilution.
4. Add 100 μ L of diluted calibrator, controls, and patient samples to the appropriate microwells.
5. Add 100 μ L of Sample Diluent to the reagent blank well. Place nothing in the well intended for the water blank.
6. Incubate 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the sample fluid. Do not allow samples to contaminate other microwells.
7. Wash **5** times with PBS. Each well should be filled with PBS per wash. Wash solution in the empty well intended to serve as a water blank well will not interfere with the procedure. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. The frame must be squeezed at the center on the top and bottom to retain microwell modules during washing. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
8. Add 100 μ L anti-IgA HRP-Conjugated Antibody Solution (orange) to the wells corresponding to the IgA calibrator, controls, reagent blank, and patient samples. Do not add conjugate to the water blank well.

9. Incubate for 15 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the conjugate solution.
10. Wash **5** times with PBS as in step 7. Wash solution in the water blank well does not interfere with the procedure. Use a snapping motion to drain the liquid and blot on absorbent paper after the final wash. Do not allow the wells to dry out.
11. Add 100 μ L One Component Substrate to each well (except for the water blank well) and incubate for 10 minutes at room temperature. Add substrate to the wells at a steady rate. Blue color will develop in wells with positive samples.
12. Add 100 μ L Stopping Solution (0.36 N sulfuric acid) to each well (except for the water blank well) to stop the enzyme reaction. Be sure to add Stopping Solution to the wells in the same order and at the same rate as Substrate was added. Blue Substrate Solution will turn yellow and colorless solution will remain colorless. Do not add Stopping Solution to the water blank well. Instead, add 200 μ L of reagent grade water to the water blank well. Blank or zero the plate reader against the water blank well. Read the O.D. of each well at 450 nm. The O.D. values should be measured within 30 minutes of the addition of Stopping Solution.

Results

1. Calculate the mean O.D. values for the duplicates of the calibrator, controls, and patient samples.
2. Divide the concentration value of the calibrator serum (printed on the vial label) by the mean O.D. value of the calibrator serum to obtain the conversion factor.
3. Multiply the mean O.D. value for each of the controls and patient samples by the conversion factor to obtain a concentration value in APL units.

$$\text{Conversion Factor} = \frac{\text{IgA anti-cardiolipin concentration of Calibrator (APL)}}{\text{Absorbance value of the Calibrator (O.D.)}}$$

$$\text{IgA anti-cardiolipin concentration of sample} = \text{Conversion Factor} \times \text{Absorbance of the sample (O.D.)}$$

4. The conversion factor must be calculated for each assay run. Using a conversion factor from another assay will invalidate the results.
5. Samples with anti-cardiolipin values greater than 80 APL may be reported as “greater than 80 APL”, or they can be further diluted and re-assayed to obtain a more accurate estimate of IgA anti-cardiolipin antibody concentration. Results from the second assay for these samples must be multiplied by the dilution factor to obtain the final IgA anti-cardiolipin value.
6. Assure that all quality control parameters have been met (see Quality Control) before reporting test results.

QUALITY CONTROL

1. The mean O.D. value of the calibrator should be at least 0.400 to assure that the kit is functioning properly. Calibrator O.D. reading of less than 0.400 may indicate that the kit is no longer suitable for use.
2. The mean O.D. of the reagent blank should be less than 0.100 when the spectrophotometer has been blanked against the water well. Readings greater than 0.100 may indicate possible reagent contamination or inadequate plate washing.
3. The anti-cardiolipin values obtained for the control sera should be within the ranges indicated on the container labels. Occasional small deviations outside these ranges are acceptable.

4. O.D. values for the duplicates of the controls or patient samples should be within 20% of the mean O.D. value for samples with absorbance readings greater than 0.200.
5. Each laboratory should periodically determine their own normal cut-off values for the appropriate population of patients. See Performance Characteristics, Clinical Specificity, as an example.

EXPECTED VALUES

Serum samples from 121 healthy blood donors were tested and the following normal range established (mean + 3 SD):

- Less than 22 APL

PERFORMANCE CHARACTERISTICS

Clinical Specificity

Normal Samples:

Serum samples from 121 healthy blood donors were assayed for the presence of IgA aCL antibodies on three separate occasions. The normal cut-off was calculated as the mean APL antibody level plus three standard deviations. The value calculated for the cut-off is 22 APL. Using this cut-off value, the assay is 95% specific for IgA antibodies.

Clinical Sensitivity

SLE:

Serum samples from 144 individuals with SLE were tested in the kit. Thirty-seven of the samples (26%) were positive for IgA anti-cardiolipin antibodies. No correlation was found between anti-cardiolipin antibody levels and anti-dsDNA antibody levels or disease activity. Monoclonal antibodies specific for dsDNA and ssDNA have also been tested in the assay and were shown to be non-reactive with the cardiolipin coated microwells.

Other Disease States:

Eighteen serum samples from patients with osteoarthritis (OA) were tested in the assay. None were positive for IgA aCL antibodies.

Ninety-two serum samples from patients with progressive systemic sclerosis (PSS) were tested in the assay. Twenty-seven of the samples (29%) were positive for IgA aCL.

The clinical significance of positive results in disease states other than SLE is still under investigation.

SLE and Thrombosis:

Serum samples from 19 patients with SLE or lupus-like disorder, known to have had at least one thrombotic event/thrombocytopenia, were tested in the assay. Fifteen samples were positive for a sensitivity of 79% in this sample population.

Disease Controls:

Serum samples from 14 patients with SLE or a lupus-like disorder with no history of thrombosis or other features of the antiphospholipid syndrome were tested in the assay. One of the samples was positive for IgA aCL antibodies. The overall specificity of the assay for this sample population was 93%.

Precision

Two samples with known APL values (one low and one high) were assayed in 26 replicates on three different occasions. The intraassay and interassay coefficients of variation (CVs) are presented in the table below. The reported intraassay coefficient of variation is the mean of the three separate intraassay CVs.

<u>Sample (aCL conc.)</u>	<u>Mean Intraassay CV</u>	<u>Interassay CV</u>
Low (16.0 APL)	4.2%	11.3%
High (33.0 APL)	8.7%	10.7%

LIMITATIONS OF THE TEST

The clinical significance of elevated anti-cardiolipin antibody levels in diseases other than SLE is still under investigation. In published studies, the reported prevalence of IgA aCL antibodies detected in SLE patients varied up to 44%.^{12, 17}

When a normal anti-cardiolipin antibody level is found in the presence of clinical manifestations, the lupus anticoagulation test should be performed.²⁰ Diagnosis cannot be made on the basis of anti-cardiolipin positive results alone. These results must be interpreted in conjunction with patient history, clinical symptoms, physical findings, and other diagnostic procedures. Treatment of patients should not be initiated on the basis of a positive anti-cardiolipin antibody result alone. Supporting clinical indications, other laboratory findings, and physician impression must be considered before any treatment is initiated.

A high percent of confirmed active or seropositive syphilis patients will have elevated anti-cardiolipin antibody levels. Confirmatory procedures should be performed to rule out syphilis in anti-cardiolipin antibody positive individuals. Anti-cardiolipin antibodies could appear transiently during many infections. If a patient tests positive for anti-cardiolipin antibodies while there are clinical signs of infection, the tests should be repeated after an appropriate interval.²¹

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Warranty

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