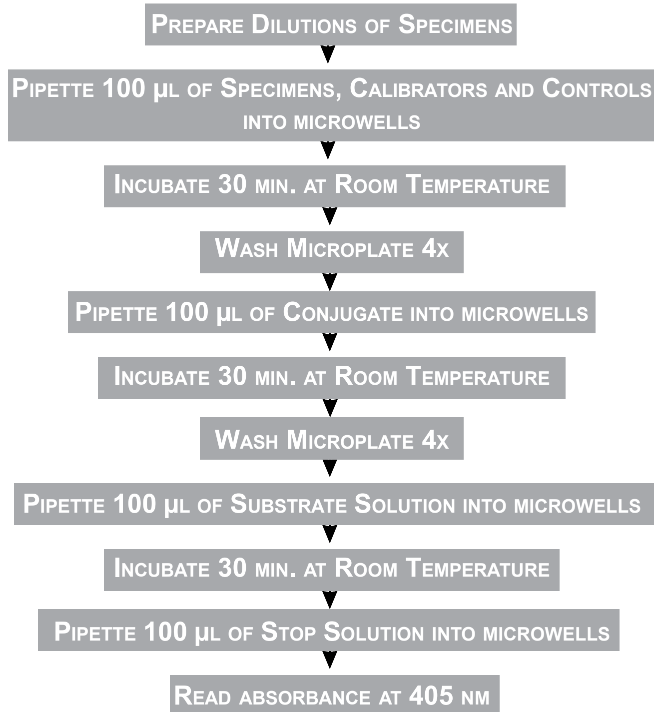
ImmuLisa™ Procedure at a Glance



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ImmuLisa™

Dual Anti-Cardiolipin Antibody (ACA) ELISA

[IVD] For *in vitro* Diagnostic Use
CLIA Complexity: High
CDC Analyte Identification Code: 0434
CDC Test System Identification Code: 28327-28328-28329

PRODUCT INSERT

Catalog No. 1118G/M **IgG-ACA/IgM-ACA** 48 Determinations

INTENDED USE

An enzyme linked immunoassay (ELISA) for the detection and semi-quantitation of IgG or IgM anticardiolipin antibodies, as an aid in assessing the risk of thrombosis in individuals with Systemic Lupus Erythematosus (SLE) or lupus like disorders.

SUMMARY AND EXPLANATION

Antiphospholipid antibodies are a heterogeneous group of autoantibodies against negatively charged phospholipids[1]. They are detected primarily by the anti-cardiolipin antibody (ACA) test, the biological false positive test for syphilis and the lupus anticoagulant test. These three tests detect related, but not necessarily identical antibodies. Thus, more than one of these tests is sometimes necessary to identify antiphospholipid antibodies.

Of the various tests for the detection of antiphospholipid antibodies, the anti-cardiolipin antibody test performed by ELISA is the most sensitive[2]. The presence of anti-cardiolipin antibodies helps to identify patients at risk of venous and/or arterial thrombosis often accompanied by thrombocytopenia, a syndrome referred to as antiphospholipid syndrome[1-12]. The antiphospholipid syndrome most commonly occurs in patients with systemic lupus erythematosus (SLE) or lupus-like disease where the criteria for SLE are not fulfilled[5-7]. High levels of anti-cardiolipin antibodies occur in thrombosis, fetal loss, thrombocytopenia and several other disorders[1-15]. Low levels of anti-cardiolipin antibodies are found in a variety of clinical disorders which are unrelated to antiphospholipid syndrome. Therefore, low levels of these antibodies are of limited significance.

IgG class anti-cardiolipin antibodies appear to be more closely associated with antiphospholipid syndrome than the IgM class antibodies. However, IgM antibodies appear to be more influenced by treatment[5,10]. Low levels of IgM antibodies can be identified in other autoimmune diseases such as rheumatoid arthritis, primary Sjögren's Syndrome, drug induced lupus erythematosus, Lyme disease, and syphilis[8,10]. The Calibrators and Positive Control used in the kit have been standardized against the Harris calibration standards from the Antiphospholipid Standardization Laboratory[16].

PRINCIPLES OF PROCEDURES

The test is performed as a solid phase immunoassay (ELISA). Microwells are coated with cardiolipin antigen followed by blocking the unreacted sites to reduce non-specific binding. Controls, calibrators and patient serum samples are incubated in the antigen coated wells which allows anti-cardiolipin antibodies present in the serum to bind. Unbound antibody and other serum proteins are removed by washing the microwells. Antibodies bound to the microwells are detected by adding an enzyme labeled anti-human IgG or IgM conjugates to the wells. These enzyme conjugated antibodies bind specifically to the human immunoglobulin of the appropriate class. Unbound enzyme-labeled conjugate is removed by washing. Specific enzyme substrate (pNPP) is then added to the wells and the presence of antibodies to cardiolipin is detected by a color change produced by the conversion of the pNPP substrate. The reaction is stopped and the intensity of color change, which is proportional to the concentration of antibody, is read by a spectrophotometer at 405 nm. Results are expressed in units per milliliter (U/ml) according to the Harris classification[16]. Units for IgG are expressed as GPL, and for IgM as MPL.

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.** Do not use if reagent is not clear or if a precipitate is present. All reagents must be brought to room temperature (20-25°C) prior to use. When stored at 2-8°C, the reconstituted wash buffer is stable until the kit expiration date. Reconstitute the wash buffer to 1 liter with distilled or deionized water. Coated microwell strips are for one time use only.

Precautions

For *in vitro* Diagnostic Use. All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by FDA required tests. However human blood derivatives and patient specimens should be considered potentially infectious. Follow good laboratory practices in storing, dispensing and disposing of these materials[17].

WARNING - Sodium azide (NaN_3) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. Sodium azide may be toxic if ingested. If ingested, report incident immediately to laboratory director or poison control center.

Instructions should be followed exactly as they appear in this kit insert to ensure valid results. Do not interchange kit components with those from other sources other than the same catalog number from IMMCO DIAGNOSTICS. Follow good laboratory practices to minimize microbial and cross contamination of reagents when handling. Do not use beyond expiration date on the label.

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Materials provided

ImmuLisa™ IgG/IgM-ACA Test System *Catalog No. 1118G/M*

Kits contain sufficient reagents to perform 48 determinations of each isotype.

6 x 8	Ready to use **breakaway** microwells coated with cardiolipin antigen for testing IgG class ACA.
6 x 8	Ready to use **breakaway** microwells coated with cardiolipin antigen for testing IgM class ACA.
1 x 1.5 ml	*Ready to use **ACA IgGPositive Control** *(red cap)*. Contains human serum positive for ACA. The expected concentration range in U/ml is printed on the label.
1 x 1.5 ml	*Ready to use **ACA IgMPositive Control** *(red cap)*. Contains human serum positive for ACA. The expected concentration range in U/ml is printed on the label.
1 x 1.5 ml	*Ready to use **Negative Control** *(white cap)*. Contains human serum.
4 x 1.5 ml	*Ready to use **set of 4 ACA IgG Calibrators**; Calibrator A *(green cap)*, Calibrator B *(violet cap)*, Calibrator C *(blue cap)* and Calibrator D *(yellow cap)*. Human serum containing antibodies to cardiolipin. Concentrations in U/ml are printed on the labels.
4 x 1.5 ml	*Ready to use **set of 4 ACA IgM Calibrators**; Calibrator A *(green cap)*, Calibrator B *(violet cap)*, Calibrator C *(blue cap)* and Calibrator D *(yellow cap)*. Human serum containing antibodies to cardiolipin. Concentrations in U/ml are printed on the labels.
1 x 12 ml	*Ready to use **anti-human Alk. Phos. IgG Conjugate**. Color coded pink.
1 x 12 ml	*Ready to use **anti-human Alk. Phos. IgM Conjugate**. Color coded pink.
1 x 60 ml	*Ready to use **Serum Diluent**. Color coded blue.
1 x 12 ml	*Ready to use **Enzyme Substrate**. Contains pNPP. **Protect from light.**
1 x 12 ml	Ready to use **Stop Solution**.
2 vials	Powder **Wash Buffer**. Reconstitute to one liter each.
1 x	Protocol Sheets
	*Contains <0.1% NaN_3

Materials Required But Not Provided

- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer
- Pipettes capable of delivering 5 µl to 1000 µl
- Disposable pipette tips

- Clean test tubes 12 x 75 mm and test tube rack
- Timer
- Absorbent paper
- Microplate reader capable of reading absorbance values at 405 nm. If dual wavelength microplate reader is available, the reference filter should be set at 600-650 nm.
- Automatic microplate washer capable of dispensing 200 µl

SPECIMEN COLLECTION AND HANDLING

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2°- 8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples.

NOTE: Must use matching set of IgG or IgM positive control, calibrators, conjugate and microwells.

PROCEDURE

Procedural Notes

- Before starting with the assay read carefully the product insert.
- Let serum specimens and test reagents equilibrate at room temperature for at least 30 minutes before starting with the test procedure. Return all unused specimens and reagents to refrigerator immediately after use.
- All dilutions of the patient samples should be prepared prior to starting with the assay.
- Good washing technique is critical. If washing is performed manually, adequate washing is accomplished by directing a forceful stream of wash buffer with a wide tip wash bottle across the entire microplate. **An automated microplate washer is recommended.**
- Use a multichannel pipette capable of delivering 8 wells simultaneously. This speeds the process and provides for a more uniform incubation time.
- For all steps, careful control of timing is important. The start of all incubation periods begins with the completion of reagent addition.
- Addition of all samples and reagents should be performed at the same rate and in the same sequence.
- Remove required microwell strips from the pouch and carefully reseal the pouch to prevent condensation in the unused wells. Return pouch immediately to refrigerator.

Test Method

Step 1 Let all reagents and specimens equilibrate at room temperature.

Step 2 Label protocol sheet to indicate sample placement in the wells. It is good laboratory practice to run samples in duplicate.

Precision:

Two samples with known concentrations of ACA were assayed in 10 replicates over a period of two weeks. Intra-and inter-assay coefficient of variation (CV) were as follows:

	GPL	MPL
Inter-assay	16.7	5.0
Intra-assay	11.0	5.6

Recovery:

Samples with known concentrations of ACA were mixed with the appropriate dilutions of another positive sample with known amounts of ACA. The IgG- and IgM-ACA values of the spiked samples were then determined and from the values obtained the percent recovery calculated.

IgG-ACL

GPL conc. added	GPL conc. obtained	% Recovery
19.5	16.5	86
9.75	8.6	88
7.8	6.9	88

IgM-ACL

MPL conc. added	MPL conc. obtained	% Recovery
29.3	26.4	90
19.5	18.3	94
14.7	12.7	87

EXPECTED VALUES

The incidence of ACA in various disease conditions is summarized in the following tables:

Incidence of ACA in SLE[15,18]

Antibody Isotype	% Incidence
IgG	39-44
IgM	5-33
Any isotype	53

Disease Association of ACA

Condition	% Incidence
Recurrent Venous Thrombosis	28-71
Recurrent Fetal Loss	28-64
Transverse Myelitis	50
Hemolytic Anemia	38
Thrombocytopenia	27-33
Arterial Occlusions	25-31
Livedo Reticularis	25
Pulmonary Hypertension	20-40

PERFORMANCE CHARACTERISTICS

Comparison of ELISA Methods for the Detection of ACA

ImmuLisa™ IgG-ACA

Other ELISA	Positive	Negative	Total
Positive	41	1	42
Negative	1	53	54
Total	42	54	96

Relative Agreement: 98%
Relative Sensitivity: 98%
Relative Specificity: 98%

ImmuLisa™ IgM-ACA

Other ELISA	Positive	Negative	Total
Positive	32	9	41
Negative	0	55	55
Total	32	64	96

Relative Agreement: 91%
Relative Sensitivity: 78%
Relative Specificity: 100%

Step 3 For a **qualitative determination** use only the Ready to Use Low Calibrator D (*vial with yellow cap*).

or For a **semi-quantitative determination** use the Ready to Use Calibrators A through D as depicted in the sample layout below.

Step 4 Prepare a **1:101** dilution of the patient samples by mixing **5 µl** of the patient sera with **0.5 ml** of Serum Diluent.

Step 5 Pipette **100 µl** of Ready to Use Calibrators, Positive and Negative controls and diluted patient samples to the appropriate microwells as per protocol sheet.

Note: Include one well which contains **100 µl** of the Serum Diluent as a reagent blank. Zero the ELISA reader against the reagent blank. The absorbance of the reagent blank should not be more than 0.3 when read against air.

Step 6 Incubate **30 minutes** (± 5 min) at room temperature.

Step 7 Wash **4x** with wash buffer. For manual washing, fill each microwell with reconstituted wash buffer. Discard the fluid by inverting and tapping out the contents of each well or by aspirating the liquid from each well. To blot at the end of the last wash, invert strips and tap the wells vigorously on absorbent paper towels. Do not dry wells completely. For automatic washers, program the washer as per manufacturer's instructions.

Step 8 Pipette **100 µl** of Conjugate into microwells.

Step 9 Incubate **30 minutes** (± 5 min) at room temperature.

Step 10 Wash all microwells as in Step 7.

Step 11 Pipette **100 µl** of Enzyme Substrate into each microwell in the same order and timing as for the Conjugate.

Step 12 Incubate **30 minutes** (± 5 min) at room temperature.

Step 13 Pipette **100 µl** of Stop Solution into each microwell using the same order and timing as for the addition of the Enzyme Substrate. Read absorbance values within 1hour from adding Stop Solution.

Step 14 Read absorbance of each microwell at **405 nm** using a single or dual wavelength microplate reader against the reagent blank set at zero absorbance.

Quality Control

Calibrators, Positive and Negative Controls and a reagent blank must be run with each assay to verify the integrity and accuracy of the test. The absorbance reading of the reagent blank should be <0.3. The Calibrator A should have an absorbance reading of not less than 1.0, otherwise the test must be repeated. The negative control must be <19 GPL for IgG, and <10 MPL for IgM. If the test is run in duplicate, the mean of the two readings should be taken for determining U/ml. While performing Qualitative determinations, the optical density of the Calibrator D must be greater than that of the negative control and lesser than the absorbance of the positive control. For semi-quantitative determinations the positive control must give values in the range stated on the vial.

RESULTS

Calculations

The concentrations of the patient samples can be determined by either of two methods:

1. **QUALITATIVE DETERMINATION**

$$\frac{\text{Abs. of Test Sample}}{\text{Abs. of Calibrator D}} \times \text{U/ml of Calibrator D} = \text{U/ml Test Sample}$$

2. **SEMI-QUANTITATIVE DETERMINATION**

Plot absorbance of Calibrator A through D against their respective concentration on a linear-linear graph paper. Plot the concentration in U/ml on the X-axis against the absorbance on the Y-axis and draw the best fit curve. Determine the concentrations of the patient samples from the curve against its corresponding absorbance value.

Calibrator

The Ready to Use Calibrators are included to provide semi-quantitation and must be used with each run. Patient samples containing higher antibody levels may give absorbance values greater than that of the Calibrator A. For determining accurate semi-quantitative values such serum sample should be further diluted so they fall within the range of the calibrator curve when retested. For determining U/ml, multiply the units obtained by the dilution factor.

Interpretation

The following serves only as a guide in the interpretation of the laboratory results. Each laboratory must determine its own normal values.

ACA values U/ml	Interpretation
IgG <19 GPL IgM <10 MPL	Negative
IgG >19 GPL IgM >10 MPL	Positive

The literature suggests that, low positive anti-cardiolipin antibody levels may occur in a variety of clinical disorders unrelated to antiphospholipid antibody syndrome. Hence according to the investigators recommendations the diagnosis of antiphospholipid antibody syndrome should be made only when the test results are moderately or highly positive[14].

LIMITATIONS OF THE PROCEDURE

The ImmuLisa™ ACA Test should not be performed on grossly hemolyzed, microbially contaminated or lipemic samples. This method should be used for testing human serum samples only.

Testing for all three isotypes of ACA is strongly recommended. Testing for only one and not all isotypes may lead to false negative results. Furthermore, a diagnosis cannot be made on the basis of ACA results alone. The results of other laboratory tests and clinical findings must be considered. Tests for rheumatoid factor (RF) should be performed because RF may interfere with this assay. When a negative ACA test occurs in the presence of clinical indications, a lupus anticoagulant test or other additional testing is indicated. ACA also occur transiently in a variety of infectious diseases. In these cases patients positive for ACA should be retested following an appropriate interval. Confirmed active or seropositive syphilis patients can have elevated ACA levels. To rule out syphilis, confirmatory tests should be performed.

Anti-cardiolipin antibodies have also been associated with neurological syndromes such as transient ischemic effects and migraine[13]. In contrast, in patients with antiphospholipid syndrome, the antibodies usually persist for longer periods and may even precede the onset of clinical symptoms[2-4].