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Anti Cardiolipin Screen

for routine analysis

Quantitative determination of IgG or IgM autoantibodies against cardiolipin in human serum or plasma

IVD

LOT
see external label

2°C 8°C

Σ = 96 test

REF DKO144

INTENDED USE

Eagle Biosciences Anti Cardiolipin Screen ELISA Assay Kit is an indirect solid phase immunoassay kit for the quantitative measurement of IgG or IgM class auto-antibodies directed against Cardiolipin-β2-glycoprotein complex in human serum or plasma. Anti Cardiolipin Screen ELISA Assay Kit is intended for research use only and not intended for diagnostic procedures.

1. CLINICAL SIGNIFICANCE

The first study on the anti-phospholipid antibodies began in 1906 when Wasserman introduced a serological test for syphilis. In 1942 it was discovered that the active component is a phospholipid called Cardiolipin. In the 1950's it was observed that a large number of people appeared to be positive for syphilis tests but did not show any evidence of disease. Initially the phenomenon was classified as a series of false positive syphilis tests, before a more accurate analysis revealed, for this group of patients, a high prevalence of autoimmune disorders including SLE and Sjögrens syndrome. The term lupus anticoagulant (LA), used for the first time in 1972, derives from experimental observations in which an increased risk of thrombosis was observed, paradoxically, with the presence of some anticoagulants factors; the term LA is not totally correct, in fact the disease is present more frequently in patients without lupus and it is associated with thrombosis rather than to abnormal bleeding.

Some years later the role of a cofactor was investigated, the β2-glycoprotein I (apolipoprotein H) also called β2GPI, and its interactions with anionic phospholipids in human serum / plasma. This cofactor is a β-globulin with a molecular weight of 50 kDa that has a concentration of 200 µg / mL in plasma. The β2GPI is involved in the regulation of blood coagulation, inhibiting the intrinsic way. β2GPI in vivo is associated with negatively charged substances such as anionic phospholipids, heparin and lipoproteins. The region that binds phospholipids is in its fifth domain. The acronym "aPL" (anti-phospholipid antibodies) indicates improperly antibodies directed against negatively charged phospholipids like Cardiolipin (CL), Phosphatidyl serine (PS) Phosphatidyl inositol (PI) and phosphatidic acid (PA); more correctly the term anti-phospholipid antibodies indicates those antibodies directed against the complex between β2GPI and anionic phospholipids that can bind to the fifth domain of β2GPI. Among these, the Cardiolipin is the most commonly used phospholipid as an antigen for determining the aPL by ELISA method. Diagnostic laboratories measure the antibodies directed against the complex between β2GPI and negatively charged phospholipids, as Phosphatidyl serine (PS) Phosphatidyl inositol (PI) and phosphatidic acid (PA). Some researchers suggest the use of PS instead of Cardiolipin in ELISA assays, for a more precise diagnosis. However, these antibodies against phospholipids are less commonly used, even if their use may increase the clinical sensitivity of patient samples with suspected

2. PRINCIPLE

Eagle Biosciences Anti Cardiolipin Screen ELISA Assay Kit allows to determine the unknown concentration of auto-antibodies directed against the Cardiolipin-β2-glycoprotein complex through two different calibration curve (one specific for IgG test, one specific for IgM test), two different conjugates linked to horseradish peroxidase (one specific for IgG test, the other specific for IgM test) and one microplate only. The principle of the method and the procedure are the same in both the tests. Use reagents for IgG or reagents for IgM depending on the isotype which is under investigation.

Anti Cardiolipin Screen ELISA Assay Kit is based on the binding of antibodies directed against the antigenic complex between Cardiolipin and β2-Glycoprotein on human serum or plasma; this complex is coated on the microplate. Through the first incubation of 60 minutes, the IgG and IgM class antibodies directed against these complexes and present in calibrators, controls and prediluted samples bind to the antigenic complexes coated on the microplate. At the end of incubation, the microplate is washed with a Wash Solution to remove the non-reactive serum or plasma components.

Then an incubation with the conjugate is performed: in this step, the anti human IgG antibodies (Conjugate IgG, reactive 3) or anti human IgM (Conjugate IgM, reactive 6) recognize the IgG or IgM class antibodies (respectively) bound to the immobilized antigenic complexes. After a 60 minutes incubation, the excess of unbound conjugate is washed away with the Wash Solution. A chromogenic substrate solution (TMB Substrate) containing tetramethylbenzidine is then dispensed into the wells. After a 15 minutes incubation, the color development is stopped by adding the Stop Solution. The solution turns into yellow and the intensity of color is directly proportional to the concentration of IgG or IgM antibodies present in the sample. The concentration of IgG or IgM class antibodies directed against the Cardiolipin-β2-glycoprotein complex in the sample is derived from a calibration curve, specific for IgG or IgM.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit

- **Reagents for IgG class antibodies assay**

1. Anti Cardiolipin IgG Calibrators
(5 vials, 1,2 mL each)
Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum

CAL0	**REF DCE002/11306-0**
CAL1	**REF DCE002/11307-0**
CAL2	**REF DCE002/11308-0**
CAL3	**REF DCE002/11309-0**
CAL4	**REF DCE002/11310-0**

2. Controls (2 vials, 1,2 mL each, ready to use)
Phosphate buffer 0,1M, NaN_3 < 0,1%, human serum

Negative Control	**REF DCE045/11301-0**
Positive Control	**REF DCE045/11302-0**

3. Conjugate IgG (1 vial, 15 mL)
Anti h-IgG conjugate with horseradish peroxidase (HRP), BSA 0,1%, Proclin < 0,0015% **REF DCE002/11302-0**

- **Reagents for IgM class antibodies assay**

4. Anti Cardiolipin IgM Calibrators
(5 vials, 1,2 mL each)
Tampone fosfato 0,1M, NaN_3 < 0,1%, siero umano

CAL0	**REF DCE002/11206-0**
CAL1	**REF DCE002/11207-0**
CAL2	**REF DCE002/11208-0**
CAL3	**REF DCE002/11209-0**
CAL4	**REF DCE002/11210-0**

5. Controls (2 flaconi, 1,2 mL ciascuno, pronti all'uso)
Tampone fosfato 0,1M, NaN_3 < 0,1%, siero umano

Negative Control	**REF DCE045/11201-0**
Positive Control	**REF DCE045/11202-0**

6. Conjugate IgM (1 flacone, 15 mL)
Anti h-IgM coniugate con perossidasi di rafano (HRP), BSA 0,1%, Proclin < 0,0015% **REF DCE002/11202-0**

- **Common reagents**

7. Sample Diluent (1 vial, 100 mL)
Phosphate buffer 0,1 M NaN_3 < 0,1%
REF DCE053-0

8. Coated Microplate
(1 breakable microplate coated with antigenic Cardiolipin/β-2-Glycoprotein complex) **REF DCE002/14403-0**

9. TMB Substrate (1 vial, 15 mL)
3,3',5,5'-tetramethylbenzidine 0,26 g/L, hydrogen peroxide 0,05% **REF DCE004-0**

10. Stop Solution (1 vial, 15 mL)
Sulphuric acid 0,15M **REF DCE005-0**

11. 10X Conc. Wash Solution (1 vial, 50 mL)
Phosphate buffer 0,2 M, proclin < 0,0015%
REF DCE054-0

3.2. Reagents necessary but not supplied
Distilled water.

3.3. Auxiliary materials and instrumentation
Automatic dispenser.
Microplate reader (450 nm).

4. WARNINGS
- This Anti Cardiolipin Screen ELISA Assay Kit is intended for research use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of Calibrators and controls for this product has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, the Calibrators and the Controls should be handled in the same manner as potentially infectious material.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents of the Anti Cardiolipin Screen ELISA Assay Kit contain small amounts of Sodium Azide (NaN_3) or Proclin 300^R as preservatives. Avoid the contact with skin or mucosa.
- Sodium Azide may be toxic if ingested or absorbed through the skin or eyes; moreover it may react with lead or copper plumbing to form potentially explosive metal azides. If you use a sink to remove the reagents, allow scroll through large amounts of water to prevent azide build-up.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H_2O_2 to directed sunlight, metals or oxidants. Do not freeze the solution.

5. PRECAUTIONS
- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents of the Anti Cardiolipin Screen ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all Anti Cardiolipin Screen ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange Anti Cardiolipin Screen ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- **WARNING: the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly;** therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, **for doses dispensed with the aid of automatic and semi-automatic devices,** before using the conjugate, it is advisable to

clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips.
For this purpose, Eagle Biosciences supplies a separate decontamination reagent for cleaning needles.

- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemeic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.

6. PROCEDURE

6.1. Preparation of the Calibrators (C_0…C_4)

The Calibrators are ready to use and have the following concentrations:

	C_0	C_1	C_2	C_3	C_4
AU/mL	0	5	10	20	80

Once opened, the Calibrators are stable 6 months at 2-8°C.

6.2. Sample Preparation

Either human serum or plasma samples can be used for the test execution. Test samples should be clear. Contamination by lipemia is best avoided, but does not interfere with this assay. Specimens may be refrigerated at 2-8°C for up to five days or stored at -20°C up to six months. Avoid repetitive freezing and thawing of serum samples. This may result in variable loss of autoantibody activity. Testing of heat-inactivated sera is not recommended.
All serum and plasma samples have to be diluted 1:100 with sample diluent, for example 10 µL of sample should be diluted with 990 µL of sample diluent.
The controls are ready to use.

6.3. Wash Solution Preparation

Dilute the contents of each vial of the buffered wash solution concentrate (10X) with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C. In concentrated wash solution it is possible to observe the presence of crystals. In this case mix at room temperature until complete dissolution of crystals is observed. For greater accuracy dilute the whole content of the bottle of concentrated wash solution to 500 mL, taking care also to transfer crystals completely, then mix until crystals are completely dissolved.

6.4. Procedure

- **Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.**
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C_0-C_4), two for each Control, two for each sample, one for Blank.

The following procedure is the same for both class IgG and IgM antibodies assay.

Reagents	Calibrator	Sample/ Controls	Blank
Use reagents for IgG or reagents for IgM depending on the isotype which is under investigation			
Calibrator C_0-C_4 (IgG or IgM)	**100 µL**		
Controls (IgG or IgM)		**100 µL**	
Diluted Sample		**100 µL**	
Incubate for 60 minutes at room temperature (22-28°C). Remove the content from each well; wash the wells three times with 300 µL of diluted wash solution.			
Conjugate (IgG or IgM)	**100 µL**	**100 µL**	
Incubate for 60 minutes at room temperature (22-28°C). Remove the content from each well; wash the wells three times with 300 µL of diluted wash solution.			
TMB Substrate	**100 µL**	**100 µL**	**100 µL**
Incubate for 15 minutes in the dark at room temperature (22-28°C).			
Stop Solution	**100 µL**	**100 µL**	**100 µL**
Shake the microplate gently. Read the absorbance (E) at 450 nm against Blank within 5 minutes.			

7. QUALITY CONTROL

- Anti Cardiolipin Positive and Negative Control should be run with every batch of samples to ensure that all reagents and procedures perform properly.
- Because the Positive and Negative Controls are prediluted, do not use procedural methods associated with dilution of specimens.
- Additional suitable control sera may be prepared by aliquoting pooled human serum specimens and storing at < -20°C.
- For best results, all of the criteria listed below must be met. If any of these are not met, the test should be considered invalid and the assay repeated:

The Positive and Negative Controls are intended to monitor for substantial reagent failure and they will not ensure precision at the assay cut-off.

This test is only valid if the optical density at 450 nm for the Positive Control as well as for the Calibrator (C_0-C_4) complies with the respective range indicated on the Quality Control Certificate enclosed in each test kit.

8. RESULTS

For Anti Cardiolipin Screen test a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed-Spline Approximation and log-log coordinates are also suitable. However a Lin-Log Plot is recommended.
First calculate the average optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

9. REFERENCE VALUES

In a normal range study with serum samples from healthy blood donors the following ranges, applicable for both IgG and IgM assay, have been established with the Anti-Cardiolipin/Vimentin Screen test:

	Anti Cardiolipin Screen (AU/mL)
Normal	< 10
Elevated	≥ 10

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacurer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.
It is recommended that each laboratory establishes its own normal and pathological ranges of seric Ab-Anti-Cardiolipin.

10. LIMITATIONS OF PROCEDURE

The presence of immune complexes or other immunoglobulin aggregates in the sample may cause an increased level of non-specific binding and produce false positives in this assay.

11. PERFORMANCE AND CHARACTERISTICS FOR IgG TEST

11.1. Precision and reproducibility

Precision and reproducibility are evaluated by two positive samples tested in two different runs with two different lots. Dispensing and washing operations were performed manually by an operator.
The results in terms of standard deviation and coefficient of variation are below:

	IgG			
Sample	1		2	
	SD	CV%	SD	CV%
Intra-assay	2,73	5,6	2,48	4,7
Inter-assay	0,15	7,3	5,69	12,8

11.2. Sensitivity

Test against a commercial reference kit, performed on 58 sera (including 23 positive sera and 35 negative sera) showed a sensitivity of 75.0%.

11.3. Specificity

Test against a commercial reference kit, performed on 58 sera (including 23 positive and 35 negative sera) showed a specificity of 70.0%.

11.4. Detection Limit

The lowest concentration of antibodies Anti Cardiolipin IgG that can be distinguished from Calibrator zero is about 0.08 AU/mL with a confidence limit of 95%.

12. PERFORMANCE AND CHARACTERISTICS FOR IgM TEST

12.1. Precision and reproducibility

Precision and inter and intra assay variation are evaluated by replicates of two positive samples tested in two different runs with two different lots.
Dispensing and washing operations were performed manually by an operator.
The results in terms of standard deviation and coefficient of variation are below:

	IgM			
Campione	1		2	
	SD	CV%	SD	CV%
Intra-assay	3,36	10,5	2,21	10,1
Inter-assay	0,20	9,3	4,17	10,3

12.2. Sensitivity

Test against a commercial reference kit, performed on 41 sera (including 18 positive sera and 23 negative sera) showed a sensitivity of 81.8%.

12.3. Specificity

Test against a commercial reference kit, performed on 41 sera (including 18 positive sera and 23 negative sera) showed a specificity > 99,9%.

12.4. Detection Limit

The lowest concentration of anti-Cardiolipin IgM that can be distinguished from Calibrator zero is 0.12 AU/mL with a confidence limit of 95%.

13. WASTE MANAGEMENT

Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY

1. Hughes G.R.V., Harris E.N., Gharavi A.E.: The Anticardiolipin Syndrome. J. Rheumat. 13, 3: 486-489, 1986.
2. Harris E.N. et al.: Evaluation of the anti-Cardiolipn antibody test: report of an international Workshop held 4 April 1986. Clin. Exp. Immunol. 68: 215-222, 1987.
3. Domke N., Siegert G.: Phospholipidantikörper und ihre klinische Bedeutung. Zeitschrift für Klinische Medizin 16: 1399-1401, 1988.
4. Pengo V. et al.: Immunological Specificity and Mechanism of Action of IgG Lupus Anticoagulants. Blood, Vol. 70. N. 1: 69-76, 1987.

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DiaMetra S.r.l. Headquater: Via Garibaldi, 18
20090 SEGRATE (MI) Italy
Tel. 0039-02-2139184 – 02-26921595
Fax 0039–02–2133354.
Manufact: Via Pozzuolo 14, 06038 SPELLO (PG) Italy
Tel. 0039-0742–24851
Fax 0039–0742–316197
E-mail: info@diametra.com

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EAGLE BIOSCIENCES, INC.
20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 FAX: 617-419-1110
www.EagleBio.com•info@eaglebio

ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction

- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)

- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers

- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within-run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation