Version 09-08/24

**User's Manual**

# Cardiolipin IgG/IgA,/IgM ELISA kit

REF **DEIA-JY2409**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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# PRODUCT INFORMATION

## Intended Use

The Anti-Cardiolipin (IgAGM) ELISA test kit provides a semi-quantitative or quantitative in vitro assay for human autoantibodies of the IgAGM class against cardiolipin in serum or plasma.

## Principles of Testing

The test kit contains microtiter strips each with 8 break-off reagent wells coated with cardiolipin. In the first reaction step, diluted patient samples are incubated in the wells. In many cases, antibodies to cardiolipin rely on a plasma protein (β2- glycoprotein I) as a cofactor for antigen recognition. The coating of the microplate and the sample buffer of this ELISA therefore contain this cofactor. In the case of positive samples, the specific IgAGM antibodies will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgAGM (enzyme conjugate) catalyzing a color reaction.

## Reagents And Materials Provided

1. **Microplate wells**, Coated with antigens: 12 microplate strips each containing 8 individual breakoff wells in a frame, ready for use --- 12 x 8. STRIPS.
2. **Calibrator 1** 120 RU/ml (IgAGM, human), ready for use Dark red 1 x 2.0 ml. CAL 1.
3. **Calibrator 2** 12 RU/ml (IgAGM, human), ready for use Red 1 x 2.0 ml. CAL 2.
4. **Calibrator 3** 2 RU/ml (IgAGM, human), ready for use Light red 1 x 2.0 ml. CAL 3.
5. **Positive control** (IgAGM, human), ready for use Blue 1 x 2.0 ml. POS CONTROL.
6. **Negative control** (IgAGM, human), ready for use Green 1 x 2.0 ml. NEG CONTROL.
7. **Enzyme conjugate Peroxidase-labelled anti-human IgAGM (rabbit)** Orange 1 x 12 ml. CONJUGATE.
8. **Sample buffer** ready for use Yellow 1 x 100 ml. SAMPLE BUFFER.
9. **Wash buffer** 10x concentrate Colorless 1 x 100 ml. WASH BUFFER 10x.
10. **Chromogen/substrate solution TMB/H2O2**, ready for use Colorless 1 x 12 ml. SUBSTRATE.
11. **Stop solution** 0.5M sulfuric acid, ready for use Colorless 1 x 12 ml. STOP SOLUTION.
12. **Test instruction** --- 1 booklet
13. **Quality control certificate** --- 1 protocol

## Materials Required But Not Supplied

1. Microplate reader capable of endpoint measurements at 450 nm; optional: reference filter at 620 nm
2. Data reduction software
3. Multi-channel dispenser or repeatable pipette for 100 µl
4. Vortex mixer
5. Pipettes for 10 µl, 100 µl and 1000 µl
6. Laboratory timing device

7. Distilled or deionised water
8. Measuring cylinder for 1000 ml and 100 ml
9. Plastic container for storage of the wash solution

This ELISA assay is suitable for use on open automated ELISA processors. Each assay has to be validated on the respective automated system.

## Storage

The test kit has to be stored at a temperature between +2°C to +8°C. The calibrators and positive control must be stored in aliquots at -20°C. Unopened, all test kit components are stable until the indicated expiry date.

## Specimen Collection And Preparation

1. **Sample material:** Human serum or EDTA, heparin or citrate plasma.
2. **Stability:** Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.
3. **Sample dilution:** Patient samples are diluted 1:201 in sample buffer. For example: dilute 5 µl serum in 1.0 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).
4. **NOTE:** Calibrators and controls are prediluted and ready for use, do not dilute them.

## Plate Preparation

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | C 2 | P 6 | P 14 | P 22 | | | C 1 | P 4 | P 12 | P 20 | | |
| B | pos. | P 7 | P 15 | P 23 | | | C 2 | P 5 | P 13 | P 21 | | |
| C | neg. | P 8 | P 16 | P 24 | | | C 3 | P 6 | P 14 | P 22 | | |
| D | P 1 | P 9 | P 17 | | | | pos. | P 7 | P 15 | P 23 | | |
| E | P 2 | P 10 | P 18 | | | | neg. | P 8 | P 16 | P 24 | | |
| F | P 3 | P 11 | P 19 | | | | P 1 | P 9 | P 17 | | | |
| G | P 4 | P 12 | P 20 | | | | P 2 | P 10 | P 18 | | | |
| H | P 5 | P 13 | P 21 | | | | P 3 | P 11 | P 19 | | | |

The pipetting protocol for microtiter strips 1-4 is an example for the semiquantitative analysis of 24 patient samples (P 1 to P 24).

The pipetting protocol for microtiter strips 7-10 is an example for the quantitative analysis of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample. The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and

minimizes reagent wastage. Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

## Reagent Preparation

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

1. **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag). Once the protective wrapping has been opened for the first time, the wells coated with antigens can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
2. **Calibrators and controls:** Ready for use. The reagents must be mixed thoroughly before use.
3. **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.
4. **Sample buffer:** Ready for use.
5. **Wash buffer:** The wash buffer is a 10x concentrate. If crystallization occurs in the concentrated buffer, warm it to 37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionized or distilled water (1 part reagent plus 9 parts distilled water). For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water. The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
6. **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue colored.
7. **Stop solution:** Ready for use.

**Warning:** Calibrators and controls used have been tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2 using enzyme immunoassays and indirect immunofluorescence methods. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the toxic agent sodium azide. Avoid skin contact.

## Assay Procedure

### INCUBATION

For semiquantitative analysis incubate calibrator 2 along with the positive and negative controls and patient samples. For quantitative analysis incubate calibrators 1, 2 and 3 along with the positive and negative controls and patient samples.

**(1st step) Sample incubation:** Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Incubate for 30 minutes at room temperature (+18°C to +25°C).

**Washing: Manual:** Empty the wells and subsequently wash 3 times using 300 µl of working strength wash buffer for each wash.

**Automatic:** Wash reagent wells 3 times with 450 µl working strength wash buffer.

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

**Note:** Residual liquid (> 10 µl) remaining in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g., less than 3 wash cycles, too small wash buffer volumes, or too short reaction times) can lead to false high extinction values.

Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

**(2nd step) Conjugate incubation:** Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgA) into each of the microplate wells. Incubate for 15 minutes at room temperature (+18°C to +25°C).

**Washing:** Empty the wells. Wash as described above.

**(3rd step) Substrate incubation:** Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for 15 minutes at room temperature (+18°C to +25°C) protect from direct sunlight.

**Stopping:** Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

**Measurement:** Photometric measurement of the colour intensity should be made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution. Prior to measuring, slightly shake the micro-plate to ensure a homogeneous distribution of the solution.

## Interpretation Of Results

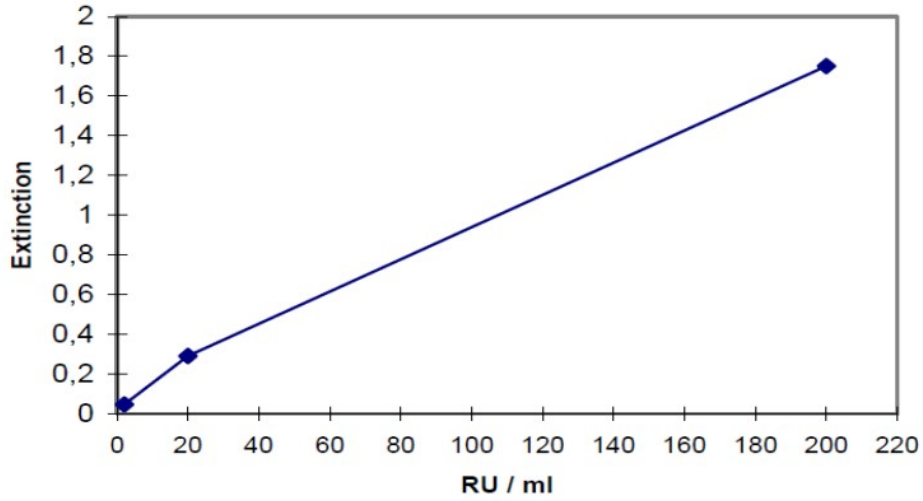
**Semiquantitative:** Results can be evaluated semi-quantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

Extinction of the control or patient sample/Extinction of calibrator 2 = Ration

Recommends interpreting results as follows:

Ratio < 1.0: Negative Ratio ≥ 1.0: Positive

**Quantitative:** The standard curve from which the concentration of antibodies in the serum samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibration sera against the corresponding units (linear/linear). Use "point-to-point" plotting for calculation of the standard curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of antibody concentrations in patient samples.




If the extinction for a patient sample lies above the value of calibrator 1 (120 RU/ml), the result should be given as ">120 RU/ml". It is recommended that the sample be re-tested at a dilution of 1:800. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range (cut-off) recommended by CD is 12 relative units (RU)/ml. CD recommends interpreting results as follows:

< 12 RU/ml: negative

**≥12 RU/ml: positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another the sample should be retested.

## Performance Characteristics

Calibration: As no international reference serum exists for the polyvalent measurement of antibodies against cardiolipin, the calibration is performed in relative units (RU). However, the controls of the Anti-Cardiolipin ELISA (IgAGM) are standardized by internationally recognized standard sera.

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratio determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: Cardiolipin is a negatively charged phospholipid which is located in high concentrations in the inner mitochondrial membrane (of nine known mitochondrial antigen types it is classified as M1). Phospholipids consist of a phosphoric acid esterified to, on the one hand, a glycerol derivative, and on the other hand, serine, choline, ethanolamine, inositol or glycerol. The glycerol derivative contains two fatty acids with double bindings of varying number and length. The glycerol derivative together with the esterified phosphoric acid form a phosphatidic acid. In cardiolipin two phosphatidic acids are linked to a further glycerol.

Antibodies against cardiolipin are probably a subtype of a class of closely related antibodies to anionic phospholipids (e.g., cardiolipin, phosphatidylserine, phosphatidylinositol) which differ in their affinities. A subpopulation of antibodies against cardiolipin (ca. 75%) relies on a plasma protein (β2- glycoprotein I, GPI) as a cofactor for antigen recognition. It is presently not clear whether this antibody population only recognizes epitopes of the GPI or also epitopes of the cardiolipin. GPI is known to interact only with anionic but not with neutral phospholipids (e.g. phosphatidyethanolamine).

## Detection Limit

1.2 RU/mL

## Specificity

Owing to the distinct structural homology of the phospholipids, antibodies to cardiolipin will cross-react with other phospholipids (phosphatidylserine, -inositol, -glycerine, -ethanolamine and -choline). No other cross-reactions are known.

## Linearity

The linearity of the Anti-Cardiolipin ELISA (IgAGM) was determined by assaying at least 4 serial dilutions of different patient samples. The Anti-Cardiolipin ELISA (IgAGM) is linear at least in the tested concentration range (8 RU/ml to 120 PL-IgA-U/ml).

## Reproducibility

The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intraassay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

| *Intra-assay variation, n = 20* | | |
|---|---|---|
| **Serum** | **Mean value (RU/ml)** | **CV (%)** |
| 1 | 23 | 11.3 |
| 2 | 60 | 7.6 |
| 3 | 91 | 10.2 |

| *Inter-assay variation, n = 4 x 6* | | |
|---|---|---|
| **Serum** | **Mean value RU/ml** | **CV (%)** |
| 1 | 25 | 8.6 |
| 2 | 63 | 7.3 |
| 3 | 93 | 7.3 |

## Interferences

Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml

for hemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.