

CE ZYMUTEST ACA-APA IgG - Isotype (# ARK029A)

Assay of Anti-Cardiolipin/Anti-Phospholipid Antibodies by ELISA



Manufactured By: HYPHEN BioMed

For in vitro diagnostic use only

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INTENDED USE:

The ZYMUTEST ACA-APA, IgG ELISA kit, is a standardised and optimised enzyme immuno-assay designed for measuring anti-cardiolipin/anti-phospholipid antibodies of the IgG isotype, in human plasma or serum, or in any biological fluid where these antibodies must be measured.

ASSAY PRINCIPLE:

The diluted assayed plasma sample or biological fluid is introduced into one of the microwells of the Cardiolipin coated plate. When present, anti-Cardiolipin/anti-phospholipid antibodies bind to immobilised and saturated Cardiolipin. Following a washing step, bound antibodies, of the IgG isotype, are revealed with a goat anti-human IgG (Fcγ specific)-peroxidase conjugate, which reacts specifically with IgG isotypes. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of hydrogen peroxide (H₂O₂), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with sulfuric acid. The colour developed is directly proportional to the amount of anti-Cardiolipin/anti-phospholipid antibodies, of the IgG isotype, present in the tested sample.

TESTED SAMPLES:

- Trisodium citrate or Na₂ EDTA anticoagulated human plasma or human serum.
- Any biological fluid, where human anti-cardiolipin / anti-phospholipid antibodies, of the IgG isotype, must be assayed.

REAGENTS:

1. **COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with anionic phospholipids, saturated, then stabilized; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
 2. **SD:** 2 vials containing 50 ml of Autoimmunity Sample Diluent, ready to use. Contains Sodium Azide
 3. **CAL:** 3 vials of Anti-Cardiolipin, IgG, calibrator, lyophilised. When restored with 1 ml of Autoimmunity Sample Diluent, the ready to use calibrator is obtained (already diluted 1:100).
- Note:** This control has a defined anti-cardiolipin concentration, expressed in GPL units (according to the KAPS standards) and indicated on the flyer provided with the kit.
4. **C₋:** 3 vials of negative control, lyophilised (diluted normal human plasma). When restored with 1 ml of Autoimmunity Sample Diluent, the ready to use negative control is obtained (already diluted 1:100).
 5. **IC:** 3 vials of immunoconjugate (Anti-IgG (Fcγ)-HRP immunoconjugate), affinity purified goat antibodies specific for human IgG-Fcγ coupled to HRP, lyophilised.
 6. **CD:** 1 vial of 25 ml of conjugate diluent, ready to use.
 7. **WS:** 1 vial of 50 ml of 20 fold concentrated Wash Solution.
 8. **TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' - Tetramethylbenzidine containing hydrogen peroxide, ready to use.
 9. **SA:** 1 vial of 6 ml of 0.45M Sulfuric Acid (Stop Solution), ready to use.

Note: Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- 8-channel or repeating pipette allowing dispensing 50-300 µl.
- 1-channel pipettes at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to 1000 µl.
- Micro ELISA plate washing equipment and shaker.
- Micro ELISA plate reader with a wavelength set up at 450 nm.
- Distilled water.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 4 weeks in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).

2. **Autoimmunity Sample Diluent:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use. This reagent contains sodium azide.

Warning: The Autoimmunity Sample Diluent contains sodium azide, which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

3. **Calibrator:** restore each vial with 1 ml autoimmunity sample diluent in order to obtain the ready to use calibrator. It corresponds to a plasma containing IgG isotype antibodies to cardiolipin, already diluted 1:100. Following reconstitution, the calibrator is stable for 5 days at 2-8°C, provided that any bacterial contamination is avoided during use.
4. **Negative control:** restore each vial with 1 ml autoimmunity sample diluent in order to obtain the ready to use negative control. It corresponds to a normal human plasma, already diluted 1:100. Following reconstitution, the negative control is stable for 2 weeks at 2-8°C, provided that any bacterial contamination is avoided during use.

Warning: The saturation solution used for coating the plates is a semi purified fraction extracted from human plasma. Calibrator and controls are also prepared with human plasma, tested with registered methods and found negative for HIV antibodies, HBs Ag and HCV antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

5. **Anti-IgG (Fcγ)-HRP immunoconjugate:** each vial must be restored with 7.5 ml of conjugate diluent. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content. The restored conjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2-8°C.
6. **Conjugate diluent:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
7. **Wash Solution:** Incubate the vial for 15-30 minutes in a water bath at 37°C until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow to prepare 1 liter of Wash Solution). The Wash Solution must be stored at 2-8°C in its original vial and used within 4 weeks following opening. The diluted Wash Solution must be used within 7 days, when protected from any contamination. This reagent contains 0.05% Kathon CG.
8. **TMB substrate:** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8°C, and provided that any bacterial contamination is avoided during use.
9. **Stop solution:** It is ready to use.

Cautions: Sulfuric Acid, although diluted to 0.45M, is caustic. As for any similar chemical, handle Sulfuric Acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

Note: Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

The stability studies performed at 30°C show that the reagents keep their performances and can be shipped at room temperature without any damage.

PROCEDURE:

Sample collection:

Blood plasma (9 vol.) must be collected on 0.109 M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within 4 hours or stored frozen at -20°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within 2 hours. EDTA collected human plasma may also be used.

Anti-cardiolipin/anti-phospholipid antibodies can also be assayed on serum.

Tested plasma or sample or control:

Plasma or serum is tested at 1:100 dilution in Autoimmunity Sample Diluent. When high amounts of anti-cardiolipin/anti-phospholipid antibodies are expected, samples must be assayed at 1:200 or 1:400 dilution, etc.... Results must then be multiplied by 2 or 4, etc....

Calibrator and negative control are ready to use (already diluted 1:100).

Assay procedure:

Calibration curve: The assay can be calibrated with the calibrator provided in the kit, and which concentration (C) is indicated in GPL units (GPL), on the flyer provided. Prepare the standard solutions for calibration by doing serial two-step dilutions of the calibrator in Autoimmunity Sample Diluent, from 1:1 to 1:32. A concentration range from C:1 to C:32 is obtained. The usual dynamic range is from 0 to about 80 GPL units.

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

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Reagent	Volume	Procedure
Anti-Cardiolipin IgG Calibrator dilutions or Negative control or 1:100 diluted sample or sample diluent (blank)	200 µl	Introduce the : – calibrator dilutions or – negative control or – diluted sample or – sample diluent into the micro ELISA plate wells (a)
Incubate for 30 minutes at room temperature (18-25 °C) (b) (c)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 5 successive washings using the washing instrument (c).
Conjugate (anti-IgG (Fc _γ)-HRP immunoconjugate, restored with 7.5 ml of conjugate diluent)	200 µl	Immediately after the washing, introduce the anti-IgG (Fc _γ)-HRP immunoconjugate in the micro ELISA plate wells.
Incubate for 30 minutes at room temperature (18-25 °C) (b)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 5 successive washings using the washing instrument (c).
TMB/H ₂ O ₂ Substrate	200 µl	Immediately after the washing, introduce the substrate into the wells. Note: The substrate distribution, row by row, must be accurate and at exact time intervals (d)
Let the colour develop for exactly 5 min. at room temperature (18-25 °C) (b)		
0.45M Sulfuric Acid	50 µl	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M Sulfuric Acid (d)
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450) (e). Subtract the blank value.		

Note:

- Distribute calibrators, controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for antibodies binding. A too long delay between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.
- Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
- For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
- For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

QUALITY CONTROL:

- Calibrator and controls provided in the kit allow validating the right performance of the assay.
- Expected A450 values for undiluted calibrator and negative controls can present variations from lot to lot but they always are:

P = A450 for 1:1 calibrator: ≥ 1.5

N = A450 for negative control: ≤ 0.25

In addition, concentrations obtained for controls must be within the acceptance ranges indicated on the flyer provided in the kit. If controls are out of these ranges check carefully the assay conditions and re-run the assay, if required.

EXPRESSION OF RESULTS:

- Results are expressed according to the A450 values obtained for samples, and controls and anti-cardiolipin/anti-phospholipid concentrations are calculated using the calibration curve.
- The calibration curve is obtained by plotting the anti-cardiolipin concentrations of calibration range expressed in GPL on the abscissae and the corresponding A450 on the ordinates. The anti-Cardiolipin, antibody concentration, of the IgG isotype, obtained for the sample tested at the standard 1:100 dilution, and expressed in GPL units, is directly deduced from the curve.
- When higher dilutions are used, (i.e. D), the concentration measured must be multiplied by the complementary dilution factor (i.e. D:100 ; for example x2 for 1:200 or x4 for 1:400), etc...
- Alternatively, an ELISA software (i.e. Dynex, Biolise, etc...), can be used for the calculation of anti-cardiolipin/anti-phospholipid antibody concentrations.

INTERPRETATION OF RESULTS:

A single and standardised calibrator is used for the assay calibration and the calibration range is prepared using a serial two-step dilutions. This ensures a higher reliability of the assay, and a higher accuracy and reproducibility from lot to lot, and run to run, for the cut-off.

Negative range: The calibrator expressed in GPL unit is defined using the KAPS standard preparation for anti-cardiolipin antibodies, IgG/IgM (N. Harris). The upper limit of the normal range corresponds to the mean value obtained in a normal population plus 2 standard deviations (SD). This corresponds to 5 GPL. Therefore:

Negative range : < 5 GPL/ml

Grey zone: A "grey zone" is defined because some pathological samples (inflammation, infectious diseases, autoimmune diseases, gammopathy, elderly people,...) can produce higher backgrounds, in auto-immune assays, than the normal individuals. This can mimic or mask a low reactivity. A value in this range can also mean that anti-cardiolipin/anti-phospholipid antibodies are being generated. When a patient is in the grey zone, it is recommended to perform a new testing on another sample, later, in order to follow a possible ongoing generation of autoantibodies to Cardiolipin of the IgG isotype.

Grey Zone: ≥ 5 GPL/ml to < 10 GPL/ml

Positive range: The positive range concerns the following anti-cardiolipin antibody concentrations:

Positive range: ≥ 10 GPL/ml

The positive range can be classified as follows:

Low positive: ≥ 10 to < 35 GPL/ml
Moderate positive: ≥ 35 to < 80 GPL/ml
High positive: ≥ 80 GPL/ml

Note: When a patient is tested positive, for the first time, for anti-cardiolipin/anti-phospholipid antibodies, a new testing or another blood specimen collected 3 to 6 months later is necessary.

LIMITATIONS OF THE ASSAY:

If the washing step is not correctly performed, the negative control can produce a high absorbance value. In order to avoid non-specific colour development, check that the washing step is performed efficiently.

As for any autoantibody assay, clinical situation such as presence of inflammation, infectious diseases, autoimmune diseases, immun-complexes, high concentrations of IgG in the tested sample, can induce a high background, which can be within the grey zone or in the weak positive range. Check then for the possible presence of antibodies on another specimen collected later.

PATHOLOGICAL VARIATIONS:

Anti-cardiolipin/anti-phospholipid antibodies are usually absent in normal population. Presence of anti-cardiolipin/anti-phospholipid antibodies at moderate or high concentrations is observed in the anti-phospholipid syndrome (APS), sometimes associated with thrombotic diseases, recurrent miscarriages, livedo reticularis, thrombocytopenia or neurological disorders. The pathogenicity of anti-cardiolipin/anti-phospholipid antibodies is still under investigation. They are thought to contribute triggering various clinical manifestations (APS). Pathogenicity of the various isotypes is not completely documented, especially for IgM and IgA isotypes. Severity of the clinical manifestations associated with the presence of anti-cardiolipin/anti-phospholipid antibodies, increases with the IgG isotype, the antibody concentration and its affinity, and the time of exposure. IgG isotype is then, the most pathogenic.

APPLICATIONS:

Assay of anti-cardiolipin/anti-phospholipid antibodies of the IgG isotype, in the following clinical situations:

- Anti-phospholipid syndrome (APS).
- Pregnancies with recurrent miscarriage.
- Unexplained thrombosis.
- Any clinical situation where the assay of anti-Cardiolipin/anti-phospholipid antibodies is required.

ASSAY SPECIFICITY AND CHARACTERISTICS:

The ZYMUTEST ACA-APA, IgG Kit, specifically measures human anti-cardiolipin/anti-phospholipid antibodies of the IgG isotype, reactive with immobilised and saturated cardiolipin. IgM or IgA isotypes are not measured. These isotypes can be assayed with ZYMUTEST ACA-APA IgM or ZYMUTEST ACA-APA IgA.

This optimised assay is designed with highly reactive cardiolipin, which has a well-controlled presentation, stabilised, and saturated. This reliable method then provides high reproducibility, high sensitivity and high specificity, and offers an optimised discrimination between normal individuals and pathologies with presence of anti-cardiolipin/anti-phospholipid antibodies.

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