APL ELISA™ IgA Kit

REF	LAPL-K-AP-A

For the Measurement of IgA Anticardiolipin Antibodies

IVD

For *In Vitro* Diagnostic Use

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INSTRUCTIONS BOOKLET

APL ELISA™ IgA Kit

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1 – INTENDED USE

The *APL ELISA™ IgA Kit* is a semi-quantitative enzyme linked immunosorbent assay (ELISA) for use as an aid in diagnosing the Antiphospholipid Syndrome (APS) in patients presenting with thrombosis, pregnancy losses and/or thrombocytopenia. It enables measurement of IgA Anticardiolipin antibody levels in human serum or plasma.

2 – EXPLANATION OF THE TEST

The anticardiolipin test (1) was devised to help in the diagnosis of patients with the Antiphospholipid Syndrome (2). The Antiphospholipid Syndrome is a disorder of recurrent venous thrombosis, pregnancy losses, and thrombocytopenia associated with positive anticardiolipin and/or lupus anticoagulant tests (3). Both the anticardiolipin and lupus anticoagulant tests detect antibodies which bind phospholipids (4, 5). These antibodies are heterogeneous, and the two tests do not necessarily identify the same antibodies (6-8). Hence, both tests should be performed in individuals suspected of having the Antiphospholipid Syndrome.

While most reports of cardiolipin antibodies center on IgG and/or IgM class antibodies (9,10), some recent studies indicate that elevated levels of IgA class cardiolipin antibodies are also found frequently in patient with SLE and related disorders (11-15). In these studies, IgA values were higher in patients with vascular complications and thrombocytopenia (13). Furthermore, studies have shown that affinity purified IgA with aCL activity are thrombogenic in mice *in vivo* (16).

The APL ELISA™ IgA Kit is calibrated using standard anticardiolipin units (APL units) and can be used to detect IgA isotype. In addition to the APL ELISA™ IgA Calibrator, an APL ELISA™ IgA Positive Control (with a defined range) and an APL ELISA™ Negative Control are included as in-house controls so that operators can determine whether a particular run is acceptable.

3 – PRINCIPLE

A standard indirect enzyme linked immunoassay (ELISA) technique has been employed in this assay. Calibrators, controls and sera are incubated in polystyrene microwell strips coated with the *APL ELISA™ Cardiolipin Antigen* and bovine β_2GPI. This process allows IgA anticardiolipin antibodies in patient sera o plasmas to react with the *APL ELISA™ Cardiolipin Antigen* associated with β_2GPI. Washing removes any unbound protein. Antibodies specific for anti-human IgA labeled with alkaline phosphatase conjugate are added. After an additional washing, a measurable color reaction ensues with the addition of an alkaline phosphatase substrate, which undergoes a color change in the presence or absence of cardiolipin antibody is determined by comparing the sample optical density with that of a five-point calibration curve. Results are reported in APL units.

4 – COMPONENTS

4.1 Contents of the *APL ELISA™ IgA Kit*

Inspect all contents of the *APL ELISA™ IgA Kit* against the list below.

	12 - *APL ELISA™ Cardiolipin Antigen* and bovine β_2GPI coated polystyrene microwell strips, 1 x 8 wells *(APL ELISA™ Plate)*	ready to use
	1 - 30 ml bottle *APL ELISA™ Sample Diluent*	ready to use. *
	1 - 40 µl vial *APL ELISA™ IgA Calibrator*	to be diluted to * prepare calibration curve as indicated.
	1 - 10 ml bottle *APL ELISA™ IgA AP Conjugate*	ready to use #
	1 - 10 ml bottle *APL ELISA™ AP Substrate*	ready to use.
	1 - 15 ml bottle *APL ELISA™ AP Stopping Solution*	ready to use.
	1 - bottle *APL ELISA™ AP PBS Concentrate*	to be diluted in * 1000 ml of dH2O.
	1 - 40 µl vial *APL ELISA™ Negative Control*	to be diluted in sample diluent. *
	1 - 40 µl vial *APL ELISA™ IgA Positive Control*	to be diluted in sample diluent. *

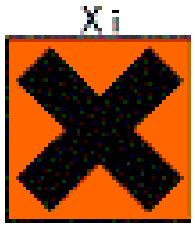
*** Contains 0.2% Sodium Azide as preservative**

 # Contains 0.05% Sodium Azide as preservative

4.2 Warnings

- This product should only be used by appropriately trained personnel.
- The use of automated systems to run the assays, to dilute samples, or to wash plates, should be validated and compared with the manual system by the user.
- Materials of human origin included in the *APL ELISA™ IgA Kit* have tested negative for HIV-I antibodies and Hepatitis B surface antigen. However, these materials and other sera to be tested should be handled as if they are infectious.
- Sodium Azide under acidic conditions yields hydrazoic acid, a very toxic compound. Azide compounds have been classified, under the directives of the European Community (CEE) as **Xn (Harmful)** and should be discarded with running water to avoid deposit in the piping system
- Avoid contact of any component of the kit with skin or mucous membranes. If an accident occurs, rinse the affected area immediately with water and consult a physician.
- The Stopping Solution contains a caustic solution (NaOH-3N). Use with care to avoid contact with skin and eyes. Avoid exposure to acids, metals, and other compounds which may react with bases. Spills should be cleaned up immediately.

R20 Harmful if inhaled.
R21 Avoid contact with skin.
R22 Harmful if swallowed.
R32 Contact with acids liberates very toxic gas.
S2 Keep out of the reach of children.
S13 Keep away from food, drink, and animal feeding stuffs.
S36 Wear suitable protective clothing.
S37 Use gloves.
S46 If swallowed, seek medical advise immediately and show this container or label.



R36: Irritating to eyes.
R38: Irritating to skin.

4.3 Required materials to run the test but not provided

- Micropipette/Multichannel pipette to deliver 5-1000 µl
- 1 liter cylinder
- Test tubes and racks
- Distilled water.
- ELISA plate reader with a 405 nm filter
- Automatic or semiautomatic ELISA plate washer (optional)
- Magnetic stirrer
- Vortex mixer

4.4 Storage and Stability

It is recommended that the *APL ELISA™ IgA Kit* be stored at 2-8 °C until expiration date, either unopened or the unused components after opening it.

- Do not freeze any of the components in the *APL ELISA™ IgA Kit.*
- Do not mix reagents between separate lots.
- Do not change any component. Substitutions will result in unreliability
- Do not use reagents beyond the expiration date.

5 – SPECIMEN COLLECTION AND STORAGE

Testing can be performed using human serum or plasma. Heat-inactivated samples (56°C for 30 minutes or more) should be avoided. Samples that are hemolyzed, lipemic or grossly contaminated should also be avoided. If patient samples will not be tested within 24 hours, they should be stored frozen at -20°C or below.

6 – INSTRUCTIONS TO USE THE KIT

6.1 Procedural Precautions

- Read instruction booklet in its entirety and review prior to testing.
- Bring all reagents and samples to room temperature before use.
- Store all unused samples in the refrigerator as soon as possible after use.
- The *APL ELISA™ IgA Calibrator* should only be used in the *APL ELISA™ IgA Kit.*
- Monitor incubation times carefully.
- Start the incubation time immediately after adding the last reagent.
- Use clean tips for each sample and reagent used.
- Pour reagents into appropriately labeled reservoirs
- Do not use Tween or other detergents, and ensure glassware is free of this agent.
- Substrate and stopping solutions must be handled carefully. Avoid contact of these solutions with skin and mucous surfaces
- Estimate the volume needed of each reagent for the run before starting. Make estimate according to the number of samples to be tested.

6.2 Detailed Procedure

a. Phosphate Buffered Saline (PBS).
- Remove the powder from the bottle labeled *APL ELISA™ AP PBS Concentrate* and add to a 1 liter cylinder.
- Add distilled water to complete 1 liter.
- Stir with a magnetic stirrer until *APL ELISA™ AP PBS Concentrate* powder is completely dissolved.
- Pour required amount into a reservoir labeled PBS, and keep at room temperature until ready for use.
- Store excess in the refrigerator.

b. Plates
- Remove the plate(s) from the pouch at least 10 minutes before use
- If the whole plate will not be used, select the strips to be used and cut the plastic cover with a sharp blade
- Separate and return unused strips to the pouch and place them in the refrigerator.
  - **After finishing the test, take out the used strips from the frame and discard them.**
  - Clean and dry the frame.
  - Reattach the unused strips to the frame. Put the frame back into the pouch and seal the pouch with tape. Store the pouch in the refrigerator.

c. Dilution of Calibrators, Samples and Controls
- Place test tubes in racks.
- Label the first column of 5 tubes, *C1* to *C5* (calibrators), label the 6th tube *P* (positive control), label the 7th tube *N* (negative control), and the 8th tube *B* (sample diluent, blank) to complete the first column of tubes.
- Label remaining tubes, U## (unknown ##), with the names of the patients and/or other samples to be tested.
- **A calibration curve should be constructed every run**
- For the calibrators:
  - Pipette 490 µl of *APL ELISA™ Sample Diluent* in tube labeled *C1*.
  - Pipette 250 µl of *APL ELISA™ Sample Diluent* to all the remaining tubes of the calibrators (C2 to C5).
  - Add 10 µl of *APL ELISA™ IgA Calibrator* (1/50 dilution) to *C1* labeled tube and vortex (avoid excessive bubbling).
  - Transfer 250 µl from C1 tube to the C2 tube, vortex and continue double dilutions through tubes C3 to C5.
- For the Positive Control, the Negative Control and unknown samples:
  - Dilute 10 µl of *APL ELISA™ IgA Positive Control* in 490 µl of *APL ELISA™ Sample Diluent.* (tube P)
  - Dilute 10 µl of *APL ELISA™ Negative Control* in 490 µl of *APL ELISA™ Sample Diluent.* (tube N)
  - For each sample, dilute 10 µl of the sample in 490 µl of *APL ELISA™ Sample Diluent.* (tube U##).
- Vortex after each dilution is made

d.. Addition of diluted calibrators, controls and samples to ELISA plates
- **All test samples, calibrators and controls should be run in duplicate.**
- Add 50 µl of the diluted calibrators in duplicate to wells labeled *C1* to *C5.*
- Add 50 µl of the diluted *APL ELISA™ Negative Control* to duplicate

wells labeled *N*.

- Add 50 µl of the diluted *APL ELISA™ IgA Positive Control* to duplicate wells labeled *P*.
- Add 50 µl of *APL ELISA™ Sample Diluent* to duplicate wells labeled *B*.
- Add 50 µl of the diluted patient samples in duplicate wells labeled *U*.
- After addition, tap the plate(s) gently once or twice to ensure even distribution.
- Incubate plate(s) for 30 minutes in a moist chamber at room temperature.

e. Washing Plates

- After incubation period, wash plates x 3 with *APL ELISA™ AP PBS*.
- This can be performed with an automatic or semiautomatic plate washer or using a multichannel pipette.
- Add 100 µl of *APL ELISA™ AP PBS* to each well for each wash.
- A reservoir should be labeled *APL ELISA™ AP PBS* for operators using a multichannel pipette. *APL ELISA™ AP PBS* can be added to this reservoir and required amounts removed as necessary.
- After each addition of *APL ELISA™ AP PBS*, tap plates gently, and then discard *APL ELISA™ AP PBS*.
- ***Make sure strips remain in place***.
- At the end of the third wash, invert plates and gently tap by turning face down on a flat area covered with blotting paper.

f. Addition of *APL ELISA™ IgA AP Conjugate*

- Carefully remove the estimated necessary amount of solution from the bottle labeled *APL ELISA™ IgA AP Conjugate* and put it into a reservoir for pipetting
- Remove 50 µl aliquots of *APL ELISA™ IgA AP Conjugate* in groups of 8 (using a multichannel pipette) and add to consecutive columns of the plates.
- After addition of the conjugate, incubate the plates covered for 30 minutes at room temperature.

g. Addition of Substrate *APL ELISA™ AP* Substrate Solution

- Preheat at 37°C for 10- 15 minutes the necessary amount of substrate solution.
- This will speed up color reaction.
- After the plates have been incubated with *APL ELISA™ IgA AP Conjugate* for 30 minutes, wash the plate x 3 with PBS as described above in paragraph ‘e’.

- Add 50 µl of *APL ELISA™ IgA AP* Substrate Solution per well, in groups of 8 or 12 using a multichannel pipette, until complete the plate.
- Put the plates in a dark box or dark container and place the plate(s) in a 37°C incubator to speed color development.
- Check the O.D. readings of the plates in the spectrophotometer until calibrator C1 reaches an O.D. reading of 1.1-1.2 (at 405 nm). This process takes approximately 15-30 minutes if incubation with substrate is done at 37°C, or up to 45 minutes if color development is done at room temperature.

h. Stopping of the color reaction

- Stop the color reaction by adding 100 µl of *APL ELISA™ IgA AP Stopping Solution* to each well, in groups of 8 or 12 using a multichannel pipette.
- Read the plate(s) **once again at 405 nm.**
- Use the data obtained to establish a calibration curve.

7 – RESULTS

7.1 Elaboration of the calibration curve

- A calibration curve should be constructed every time.
- Determine mean optical density (O.D.) reading of the calibrators (C1 to C5), positive control (P) and reagent blank (B).
- Subtract the mean O.D. readings of reagent blank (B) from all mean readings.
- Plot mean O.D. of *C1 – C5* against appropriate concentration using a log-log (Figure 1) or a log-logit (Figure 2) calibration plot.
- This is best done using a computer with appropriate software.
- The initial concentration of **the calibrator** is listed on the calibrator label.

Figure 1: Example of a calibration curve for *IgA aCL* antibodies using a log-log plot.

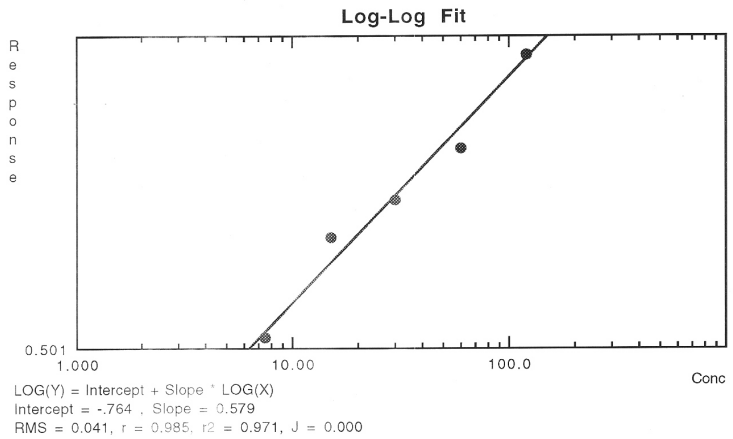
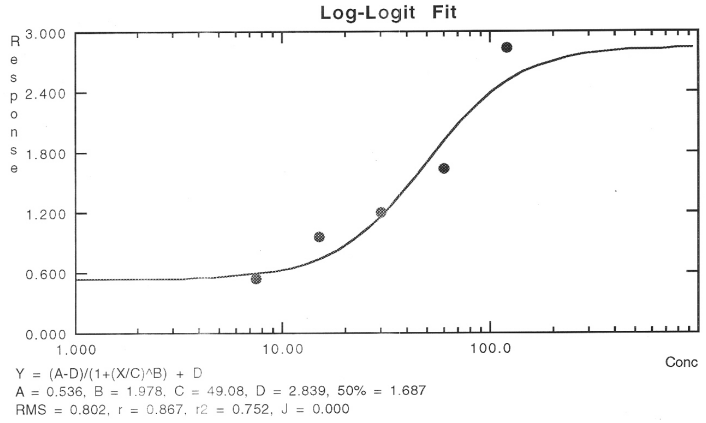


Figure 2: Example of a calibration curve of *IgA aCL* antibodies using a log-logit plot.



7.2 Example of a calibration curve.

The O.D. values obtained from a typical run is found in Table 1

- Do not use these values to construct a calibration curve.
- **This is an example only.**
- **O.D. values obtained for the given run should be used.**

Table 1. Calibration Curve Typical Values.

	Typical Curve *IgA APL*	
Calibrator	**O. D.**	**APL**
APL ELISA™ IgA Calibrator (C1)	1.014	120
APL ELISA™ IgA Calibrator (C2)	0.544	60
APL ELISA™ IgA Calibrator (C3)	0.323	30
APL ELISA™ IgA Calibrator (C4)	0.167	15
APL ELISA™ IgA Calibrator (C5)	0.078	7.5
APL ELISA™ IgA Positive Control	0.485	In range
APL ELISA™ Negative Control	0.101	<15 APL

APL: 1 APL unit is the anticardiolipin binding activity of 1µg/ml of an affinity purified IgA antibody.

7.3 Expected results

- The range within which the *APL ELISA™ IgA Positive Control* should fall is indicated in the label.
- If the *APL ELISA™ IgA Positive Control* falls outside the range indicated in the label, the operator should review the calculations and procedure for errors. If there are no apparent errors, the assay should be repeated.
- The *APL ELISA™ Negative Control* should give values lower than the suggested cut-off points of 15 APL units.
- Values lower than 27 MPL and above the cut-off point for IgA are considered “indeterminate (Grey Zone)”.
  Samples falling in this category should be retested to confirm positivity at a later date. (17)
- If a patient sample has a higher O.D. reading than calibrator C1, the sample should be serially diluted and tested again. The values obtained in APL units should be multiplied by the appropriate dilution factor(s).

8 – QUALITY CONTROL

- The *APL ELISA™ IgA Positive Control* and the *APL ELISA™ Negative Control* have been provided to help ensure that the assay is performing correctly.
- The *APL ELISA™ IgA Positive Control* has a defined IgA anticardiolipin level. Its range is indicated in the label of the vial.
- The assay is considered to be performing correctly when the IgA anticardiolipin level of the *APL ELISA™ IgA Positive Control* falls within the defined range.
- The *APL ELISA™ Negative Control* should give values lower than the suggested cut-off points of 15 APL units.
- The net O.D. of the highest calibrator should be ≥ 1.0
- ***The mean O.D. of reagent blank should be less than 0.2.***

9 – LIMITATIONS

- Diagnosis of the Antiphospholipid Syndrome cannot be based solely on a positive antiphospholipid antibody test.
- Criteria for this diagnosis include a history of one of the following clinical features: thrombosis, pregnancy loss or thrombocytopenia, combined with a positive anticardiolipin/antiphospholipid ELISA test and/or positive lupus anticoagulant test.
- Patients may have positive lupus anticoagulant but negative anticardiolipin/antiphospholipid tests; hence, both tests should be performed in patients suspected of having the Antiphospholipid Syndrome.
- In addition, a variety of infectious states (including HIV positive patients) and drug-induced disorders may yield false positive tests.

10 – CHARACTERISTICS OF THE ASSAY

10.1 Specificity

Normal

Samples from 80 normal healthy donors were tested in the *APL ELISA™ IgA Kit.* A cut-off value of 15 APL units was determined based on 3/80 gave readings >15 APL units and 77/80 gave readings <15 APL

Disease

Positive samples from different diseases were tested in the *APL ELISA™ IgA Kit.* The values obtained are listed in the following table.

Sample	Number of samples tested.	Number of samples positive *
APS and SLE	42	26
Syphilis +	33	0
Rheumatoid Factor	37	1

* Positive is defined as greater than 15 APL units for *IgA aCL*

10.2 Sensitivity

- Sera from 42 patients defined with the Antiphospholipid Syndrome were tested using the *APL ELISA™ IgA Kit.*
- 26 patients tested positive for IgA anticardiolipin antibodies.
  (Note: 24 patients tested positive for at least one other isotype IgG and/or IgM. 2 patients tested positive only for IgA aCL).

10.3 Precision

Intra-assay Variations

Intra-assay variations were determined by running 3 samples for *IgA aCL* antibodies in the *APL ELISA™ IgA Kit,* 12 times in the same plate.
Statistics were calculated and are shown in the following table.

Sample	Mean	Standard deviation	% Coefficient of Variation
A	63.0	2.5	3.9
B	32.9	3.7	11.2
C	19.3	2.2	11.4

10.4 Reproducibility

Inter-assay Variations

Inter-assay variations were determined by testing 3 positive samples (high, medium and low) samples for *IgA aCL* antibodies on the *APL ELISA™ IgA Kit* on 17 different runs.
Statistics were calculated and are shown in the following table.

Sample	Mean	Standard deviation	% Coefficient of Variation
A	117.6	14.7	12.4
B	36.8	6.7	18.2
C	16.5	3.0	18.3

10.5 Recovery

- The *APL ELISA™ AP IgA Calibrator* was diluted with normal serum as indicated in the table and the diluted samples run in the *APL ELISA™ IgA Kit*.
- The expected values, in APL units, were calculated by dividing the concentration of the *APL ELISA™ AP IgA Calibrator* by the dilution factor.
- The observed values, in APL units, were determined from the calibration curve.

Calibrator Dilution	Observed APL	Expected APL	% of Recovery
Neat	121.0	120.0	101
1:2	62.0	60.0	103
1:4	31.0	30.0	103
1:8	15.0	15.0	100
1:16	7.0	7.5	93
1:32	3.4	3.75	91

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