The PLAC® Test ELISA Kit

REF
90123

Enzyme Immunoassay for the Quantitative Determination of Lp-PLA_2 in Human Plasma and Serum

www.plactest.com

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Symbol Key

Symbol	Meaning	Symbol	Meaning
REF	Catalog Number	IVD	*In vitro* diagnostic medical device
PLATE	Antibody Coated Plate	LOT	Batch
CAL	Calibrator	(hourglass)	Expiration Date
CONTROL LOW	Control Low	2°C–8°C	Store at 2 to 8 °C
CONTROL HIGH	Control High	[Xi]	Irritant
WASH	Wash Buffer	(i)	Consult Instructions for Use
CONJ	Conjugate	(factory)	Manufacturer
TMB	TMB Substrate	EC REP	Authorized Representative in the European Community
STOP	Stop Solution	CE	European Conformity

Read this package insert completely before using the product. Follow instructions carefully when performing tests. Failure to follow the instructions may result in inaccurate results.

This product is covered by U.S. Patent Nos. 5532152, 5641669, 5698403, 5847088, 5968818, 5981252, 6177257, 7045329, 7416853 and European Patent Nos. 658205 and 673426. Additional patents pending.

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P/N 30035-05
2011-11-03

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INTENDED USE

The diaDexus PLAC® Test ELISA Kit is an enzyme immunoassay for the quantitative determination of Lp-PLA_2 (lipoprotein-associated phospholipase A_2) in human plasma and serum, to be used in conjunction with clinical evaluation and patient risk assessment as an aid in predicting risk for coronary heart disease, and ischemic stroke associated with atherosclerosis.

SUMMARY AND EXPLANATION

Lp-PLA_2 is a calcium-independent serine lipase that is associated with both low-density lipoprotein (LDL) and, to a lesser extent, high-density lipoprotein (HDL) in human plasma and serum [1] and is distinct from other phospholipases such as cPLA_2 and sPLA_2 [2]. Lp-PLA_2 is produced by macrophages and other inflammatory cells and is expressed in greater concentrations in advanced atherosclerotic lesions than early-stage lesions [3,4]. Several lines of evidence suggest that oxidation of LDL plays a critical step in the development and progression of atherosclerosis [5,6]. Lp-PLA_2 participates in the oxidative modification of LDL by hydrolyzing oxidized phosphatidylcholine, generating lysophosphatidylcholine and oxidized free fatty acids, both of which are potent proinflammatory products that contribute to the formation of atherosclerotic plaques [7,8,9]. Lp-PLA_2 has demonstrated modest intra- and inter-individual variation, commensurate with other cardiovascular lipid markers and substantially less than C-reactive protein (CRP). In addition, Lp-PLA_2 is not elevated in systemic inflammatory conditions, and may be a more specific marker of vascular inflammation. The relatively small biological variation of Lp-PLA_2 and its specificity are of value in the detection and monitoring of cardiovascular risk [10,11].

Elevated levels of Lp-PLA_2, as measured by immunoassay, were found in patients with angiographically proven coronary heart disease (CHD), when compared to age matched controls [1]. In a retrospective case-control study, using samples from hypercholesterolemic men (n=1740) in the West of Scotland Coronary Prevention Study (WOSCOPS), a 2-fold greater risk of coronary heart disease was observed for subjects in the highest quintile of Lp-PLA_2 levels, compared to the lowest quintile [12]. Furthermore, the CHD risk association of Lp-PLA_2 was shown to be independent of LDL and other markers of inflammation: C-reactive protein, white cell count and fibrinogen. The authors of the study stated in their conclusions, “Elevated levels of lipoprotein-associated phospholipase A_2 appear to be a strong risk factor for coronary heart disease, a finding that has implications for atherogenesis and the assessment of risk.” In another report, using samples from the Atherosclerosis Risk in Communities (ARIC) study, which followed 12,819 apparently healthy middle-aged (45 to 64 years) men and women for six to eight years, Lp-PLA_2 was found to be an important predictor of CHD risk. For individuals with LDL less than 130 mg/dL, Lp-PLA_2 was significantly and independently associated with a 2-fold higher risk for CHD events including the need for revascularization, myocardial infarction and death from cardiac disease [13].

The ARIC study was re-analyzed to determine the risk of stroke associated with increased levels of Lp-PLA_2. A total of 223 stroke events were identified from the study group; of this, 194 (87%) were ischemic stroke associated with atherosclerosis, as classified by the ARIC investigators. This proportion of ischemic stroke to the total is consistent with the percentage found in the general population [14]. The results of this study indicated that Lp-PLA_2 was a strong predictor of ischemic strokes, with an increased risk of nearly 2-fold, even after adjustment for blood pressure, lipids, diabetes, body mass index and other inflammatory markers [15].

PRINCIPLE OF THE TEST

The diaDexus PLAC Test ELISA Kit is a sandwich enzyme immunoassay that uses two highly specific monoclonal antibodies for the direct measurement of Lp-PLA_2 concentration in human plasma and serum. The assay system utilizes monoclonal anti-Lp-PLA_2 antibody (2C10) directed against Lp-PLA_2 for solid phase immobilization on the microwell plate. Sample is added to the plate and incubated for 10 minutes at 20 to 26 °C. A second monoclonal anti-Lp-PLA_2 antibody (4B4) labeled with the enzyme horseradish peroxidase (HRP) is then added and reacted with the immobilized antigen at 20 to 26 °C for 180 minutes, resulting in the Lp-PLA_2 molecules being captured between the solid phase and the enzyme-labeled antibodies. The wells are washed with a supplied buffer to remove any unbound antigen. The substrate, tetramethylbenzidine (TMB), is then added and incubated at 20 to 26 °C for 20 minutes, resulting in the development of a blue color. Color development is stopped with the addition of Stop Solution, changing

the color to yellow. The absorbance of the enzymatic turnover of the substrate is determined spectrophotometrically at 450 nm and is directly proportional to the concentration of Lp-PLA_2 present. A set of Lp-PLA_2 Calibrators is used to plot a standard curve of absorbance versus Lp-PLA_2 concentration from which the Lp-PLA_2 concentration in the test sample can be determined. Two levels of Controls are provided to monitor the assay performance within the clinical range of the assay.

REAGENTS AND MATERIALS

Materials supplied with the kit: (Sufficient for 96 wells)

PART NUMBER	SYMBOL	COMPONENT DESCRIPTION	QUANTITY
60001	PLATE	**Antibody Coated Plate** Mouse monoclonal anti-Lp-PLA_2 (2C10) antibody coated microwell plate	**1**
60006 **60007** **60008** **60009** **60010** **60011**	CAL	**Calibrators** (0, 50, 100, 250, 500 and 1000 ng/mL) diaDexus recombinant Lp-PLA_2 antigen (DDX-RA) in a protein stabilizing diluent	**1 set, 0.25 mL each**
60002	WASH	**20X Wash Buffer** Non-ionic detergent in a buffered solution	**50 mL**
60003	CONJ	**Conjugate** Mouse monoclonal anti-Lp-PLA_2 (4B4) antibody conjugated to horseradish peroxidase in a buffered reagent with bovine and murine carrier proteins	**23 mL**
60004	TMB	**TMB Reagent** 3,3',5,5'-tetramethylbenzidine in a mildly acidic buffer	**11 mL**
60005	STOP	**Stop Solution** 1N HCl	**11 mL**
65009	CONTROL LOW	**Control Low** (~150 ng/mL) diaDexus recombinant Lp-PLA_2 antigen (DDX-RA) in a liquid, protein (BSA) buffered matrix	**1 bottle, 0.5 mL**
65010	CONTROL HIGH	**Control High** (~350 ng/mL) diaDexus recombinant Lp-PLA_2 antigen (DDX-RA) in a liquid, protein (BSA) buffered matrix	**1 bottle, 0.5 mL**
FMD-01-026		**Certificate of Analysis – Control Range** The control ranges for the lot are indicated on the Certificate of Analysis	**1 each**

Materials required but not provided:

- Precision single and multi-channel pipettors: 0.02, 0.10, 0.20 mL
- Disposable pipette tips (a new pipette tip must be used for each addition of different samples or reagents during the assay procedure)
- Vortex mixer or equivalent
- Deionized water
- A microwell plate reader with a bandwidth of 10 nm or less and an optical density (O.D.) range of 3 or greater at 450 nm
- Computer software capable of point-to-point curve fit for calculating concentration of analyte from optical density (optional)

WARNINGS AND PRECAUTIONS

- For *In Vitro* Diagnostic Use.
- Treat all blood samples as potentially biohazardous material.
- Exposure of samples to room temperature should be minimized to less than 6 hours (including blood draw, processing, transport time and laboratory sample analysis). This does not include incubation on the ELISA plate.
- Storage of samples at -20 °C for longer than 24 hours is not recommended.
- Certain components are labeled with safety precautions. See the Product Safety Information section.
- Dispose of reagents in a manner consistent with relevant regulations.
- Do not use reagents past their expiration dates.
- Do not mix reagents from different kit lot numbers.
- If there is evidence of contamination, do not use.
- Hemolysis may affect results. Do not test hemolyzed samples.
- It is recommended that both Low and High Controls be included in each run. If control values are not within acceptance limits, repeat the assay. Additional quality control testing may be necessary according to state and local requirements.
- To avoid erroneous results, store the material as indicated.
- The control ranges provided were derived from replicate testing of the specific control lots using The PLAC Test ELISA Kits and should be used as guides. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences or reagent variability. It is recommended that each laboratory establish its own acceptable ranges.

REAGENT PREPARATION AND STORAGE

Store unopened test kits at 2 to 8 °C upon receipt. In addition, keep the microwell plate sealed in the foil pouch with desiccant to minimize exposure to moisture. Opened test kits will remain stable until the expiration date shown, provided they are stored as described above.

Prepare 1X Wash Buffer by diluting 20X Wash Buffer 1:20 with deionized water. Mix 1 part of Wash Buffer to 19 parts of deionized water. Store at room temperature (20 to 26 °C). Use 1X Wash Buffer within four weeks of preparation. If microbial growth is seen, discard.

SPECIMEN COLLECTION AND STORAGE

- Fasting is <u>not</u> required.
- Collect blood samples in
  - serum or plasma gel separation tubes
  - EDTA or heparin plasma collection tubes
  - any serum collection tubes.
- Process samples using standard separation procedures.
  - Samples should be centrifuged and separated within four hours of venipuncture as per good laboratory practices, but no longer than 36 hours after blood draw. Samples must be stored refrigerated (2 to 8 °C).
- Unprocessed blood samples:
  - Store and transport on cold packs (at 2 to 8 °C) and process within 36 hours of collection.
- Processed samples:
  - Samples must be stored refrigerated at 2 to 8 °C for a minimum of 24 hours after the sample is drawn before testing can occur.
  - Samples can be tested up to 7 days after the sample is drawn when stored at 2 to 8 °C.
  - For longer term storage, serum/plasma samples must be stored at or below -70 °C. Once thawed, the sample can be tested up to 7 days when stored at 2 to 8 °C. Samples may be frozen and thawed twice without affecting the Lp-PLA_2 quantitation.

ASSAY PROCEDURE

Calibration

Each plate or strip run must be calibrated using a full (6-point) calibration curve. A standard curve is generated using a point-to-point curve fit model. Verify the calibration curve with at least two levels of Controls according to the laboratory's requirement. Calibrate and run Controls for each plate run.

Quality Control

Test the High and Low Controls with each run. If control values are not within acceptance limits, repeat the assay. Additional quality control testing may be necessary according to local, state and/or federal regulations or accreditation requirements.

Preparatory Steps

1. Bring the microwell plate, Conjugate, Wash Buffer and TMB to room temperature (20 to 26 °C) before use.
2. Remove the plate frame and the required number of coated microwell strips from the foil pouch. Completely reseal the foil pouch containing any unused strips with the desiccant that came in the pouch and store at 2 to 8 °C.
3. Prepare 1X Wash Buffer by diluting 20X Wash Buffer 1:20 with deionized water (1 part Wash Buffer and 19 parts of deionized water). Store at room temperature (20 to 26 °C). Use 1X Wash Buffer within four weeks of preparation.
4. Allow patient samples to thaw at 2 to 8 °C, if needed, and place on ice or at 2 to 8 °C as soon as thawed.
5. Store the Controls at 2 to 8 °C or on ice until used.
6. Vortex the samples and Controls to mix thoroughly. Avoid foaming.

Sample Incubation

1. Using a pipettor and tip with appropriate low volume precision, dispense 20 µL of Calibrators, samples and Controls into the appropriate wells after vortexing. Use a calibrated pipette and new pipette tip for each Calibrator, Control or sample.
2. Allow the samples to incubate on the plate for 10 ± 2 minutes before adding the Conjugate.
3. Pipette 200 µL of room temperature Conjugate into the appropriate wells of the coated microwell plate. Avoid contamination by adding the Conjugate without touching the samples with the pipette tips. If there is cross over, change tips and continue adding Conjugate to the wells.
4. Incubate for 3 hours at room temperature.
5. At the end of the incubation period, wash the microwells four (4) times with at least 300 µL of the supplied room temperature 1X Wash Buffer. (DO NOT USE TAP or DISTILLED WATER.)
6. Blot the plate on absorbent paper after the final wash. Immediately (in less than 2 minutes) proceed to the next step. Do not allow the microwell plate to dry.

Substrate Incubation

1. Pipette 100 µL of room temperature TMB Reagent into each well.
2. Gently swirl the plate on a flat surface for 10 to 15 seconds to ensure mixing.
3. Incubate the plate at room temperature for 20 minutes in the dark.
4. Stop the reaction by adding 100 µL of room temperature Stop Solution to each well.
5. Gently swirl the plate on a flat surface for 20 to 30 seconds to ensure mixing. <u>It is important to make sure that the blue color completely changes to yellow color.</u>
6. Wipe moisture from the bottom of the plate using a paper towel.
7. Within 15 minutes of adding the Stop Solution, read the optical density (O.D.) at 450 nm using a microwell plate reader.

PROCEDURAL NOTES

- Store all test reagents at 2 to 8 °C. Except for the Calibrators and Controls, allow the reagents to equilibrate to room temperature prior to use. A 23 mL bottle of reagent may require an hour or more to reach room temperature. Keep Calibrators and Controls at 2 to 8 °C or on ice until used.
- Bring the microwell plate to room temperature before opening the bag. Store the strips in the foil pouch with desiccant to minimize exposure to moisture. Always keep the unused microwell strips in the foil pouch with desiccant.
- Always have the next step reagent ready 2 to 3 minutes before the washing step.
- For accurate measurement of samples, the addition of samples, Calibrators and Controls must be precise. Pipette carefully using only calibrated equipment.
- This assay may be performed using any validated washing method.
- Do not use plate sealers during incubations.
- Do not use a plate shaker for incubation steps.

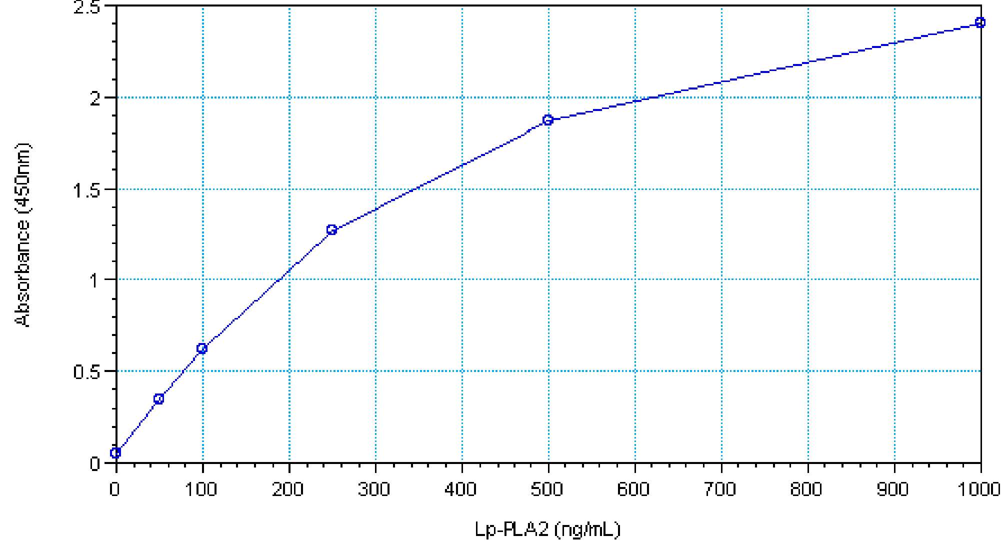
CALCULATION OF RESULTS

1. Construct a standard calibration curve by plotting the absorbance obtained for each Calibrator on the y-axis versus the Lp-PLA_2 concentration in ng/mL on the x-axis. Use a point-to-point curve fit with appropriate computer software to construct the standard calibration curve.
2. Using the absorbance value for each sample and Control, determine the corresponding concentration of Lp-PLA_2 in ng/mL from the calibration curve.

EXAMPLE OF CALIBRATOR CURVE

Results of a typical standard calibration curve with O.D. readings at 450 nm are shown on the y-axis against Lp-PLA_2 concentrations (ng/mL) shown on the x-axis. This calibration curve is for the purpose of illustration only. A standard calibration curve should be generated by the user for each assay performed.

Lp-PLA_2 (ng/mL)	**Absorbance (O.D. at 450 nm)**
0	0.048
50	0.348
100	0.623
250	1.269
500	1.868
1000	2.402



LIMITATIONS

Procedure

- Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
- The wash procedures are critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
- As with any immunoassay system, particularly those employing mouse monoclonal antibodies, the possibility exists for interference by human anti-mouse antibodies (HAMA) or other heterophilic interferences present in the sample that could cause falsely elevated or depressed values.
- As with any analytical method, the possibility exists that substances and/or factors not tested (e.g. technical or procedural) may interfere with the test and cause false results. Results should be considered in conjunction with other clinical and analytical methods.

Clinical Interpretation

- Lp-PLA_2 levels should be interpreted in conjunction with clinical findings and other diagnostic tests.
- This test does not replace blood cholesterol tests or other traditional risk factors identified for coronary heart disease or ischemic stroke.

EXPECTED VALUES

Samples from apparently healthy males (n=251) and apparently healthy females (n=174), in the clinically relevant age range of 40 to 70 years, were evaluated with the diaDexus PLAC Test ELISA Kit. The reference population was represented by the following ethnic backgrounds: African-American n=26, Caucasian n=390, Hispanic n=8 and not specified n=1. The distributions of Lp-PLA_2 values across the entire population and divided by gender appear in the following table:

	Lp-PLA_2 ng/mL		
Percentile	**All (n=425)**	**Females (n=174)**	**Males (n=251)**
5	126	120	131
20	174	169	179
33	201	188	205
50	235	228	244
67	262	252	268
80	289	285	293
95	369	342	376

These reference ranges are provided as guidelines only and are not intended to address "critical values" or medical decision limits. Each laboratory should establish its own reference intervals. Guidance for establishing reference intervals can be found in CLSI Standard C28-A2 (*How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline - Second Edition*). Based on the median population Lp-PLA_2 concentration of 235 ng/mL, it has been suggested to use that value as a clinical decision threshold [16]. More recently, an expert consensus panel has suggested applying a decision threshold of 200 ng/mL, based on a significant body of published literature for Lp-PLA_2 risk assessment [17].

PERFORMANCE CHARACTERISTICS

Sensitivity

The minimum detection limit is 0.34 ng/mL, as calculated by interpolation of the mean plus two standard deviations of 20 replicates of the 0 ng/mL Lp-PLA_2 calibrator.

Assay Precision

Intra-assay and inter-assay variability were determined by testing four human serum pools with Lp-PLA_2 concentrations distributed throughout the calibration range of the assay. The serum pools were assayed using a single lot of reagents, in duplicate, on two separate stripwells per day, for 5 days, four plates per day. The data are summarized below:

Serum Pool	Mean Concentration Lp-PLA_2 (ng/mL)	Intra-assay % CV n=80	Inter-assay % CV n=20	Total % CV n=80
1	143	6.2	4.6	7.7
2	211	4.1	5.1	6.6
3	368	5.1	8.5	9.9
4	830	9.5	8.7	12.8

In a repeatability study conducted with a set of 108 serum samples, assay results determined from the first wells (single point assay) were compared to the mean results from two successive measurements (duplicate wells). The Lp-PLA_2 levels of the samples ranged from 87 to 575 ng/mL, and the mean %CV between replicates was 2.3%. In linear regression analysis, single point results were highly correlated to duplicate mean results: correlation coefficient r=0.997 (slope 1.0 and intercept -1.3 ng/mL).

Linearity

Six serum samples with known high Lp-PLA_2 levels were intermixed with six serum samples with known low Lp-PLA_2 levels. Percent recovery was determined as the measured value divided by the expected value, multiplied by 100. The average recovery was 93%, demonstrating linearity of the diluted samples over a range of 151 to 810 ng/mL Lp-PLA_2.

Interfering Substances

Endogenous substances found in blood and exogenous substances (common and prescription drugs) were evaluated for interference in the assay. Five individual serum samples with Lp-PLA_2 values ranging from 163 to 908 ng/mL were spiked with potential interferents. No appreciable interference was observed for the following substances at the spiked levels tested.

Endogenous

Potential Interferent	Test Concentration
Bilirubin	20 mg/dL
Cholesterol	500 mg/dL
Hemoglobin	1250 mg/dL
Triglycerides	3000 mg/dL
Total Albumin*	~6500 mg/dL

* 2.5 g/dL albumin added to plasma pool of presumptively 4 g/dL albumin

Exogenous (OTC Drugs, etc.)

Potential Interferent	Test Concentration
Acetaminophen	1.66 µmol/L
Aspirin	3300 µmol/L
Atorvastatin	20 µmol/L
Clopidogrel bisulfate	140 µmol/L
Diphenhydramine	19.6 µmol/L
Fenofibrate	125 µmol/L
Lisinopril	0.74 µmol/L
Metformin	310 µmol/L
Niacin	6500 µmol/L
Pravastatin	10 µmol/L
Tolbutamide	2400 µmol/L
Vitamin C	227 µmol/L
Warfarin	64.9 µmol/L

CLINICAL STUDIES

Coronary Heart Disease

To determine the efficacy of the diaDexus PLAC Test ELISA Kit as a predictor of risk for coronary heart disease (CHD), Lp-PLA_2 levels were measured in 1348 banked EDTA-plasma samples from a large, multi-center epidemiology study, the Atherosclerosis Risk In Communities (ARIC) study, sponsored by the National Institutes of Health's National Heart, Lung, and Blood Institute. Participants were followed for the development of CHD for six to eight years. Samples used for the PLAC Test were from participants, age 47 to 69, who were free of CHD at the time of blood drawn. This was a case-cohort study where samples from all the CHD cases (607) were tested together with 741 appropriately matched participants without CHD at the time of censor (controls).*

Cox regression models were used to evaluate the association of Lp-PLA_2 and CHD in a univariate analysis (Model 1), a univariate analysis adjusted for demographics (Model 2), and a multivariate model adjusted for demographics and other prognostics factors (Model 3). Using high and low tertile cutpoints of Lp-PLA_2, generated from the ARIC data set (420 and 310 ng/mL, the 67th and 33rd percentiles, respectively), the hazard ratios of the Cox regression analyses demonstrated that Lp-PLA_2 was a significant predictor of risk for CHD, for the highest and intermediate levels when compared to the lowest level of Lp-PLA_2, for all participants (see Table 1). It should be noted that different cutpoints may be appropriate for different clinical populations.

** NOTE: 86 results (5.5%) were outside the assay acceptance criteria and were excluded from data analyses.*

Table 1. Risk Ratios of CHD for Subjects Across All LDL Levels

	Lp-PLA_2 Risk Ratio (95% CI, p value)*		
Lp-PLA_2	**Tertile 1**	**Tertile 2**	**Tertile 3**
#CHD cases/total subjects in category	127/366 **(34.7%)**	192/444 **(43.2%)**	288/538 **(53.5%)**
Model 1	**1.0**	**1.49** (1.11 to 1.99, p=0.008)	**2.50** (1.89 to 3.31, p<0.001)
Model 2	**1.0**	**1.24** (0.92 to 1.66, p=0.154)	**1.76** (1.32 to 2.36, p<0.001)
Model 3	**1.0**	**1.71** (1.06 to 2.75, p=0.029)	**2.12** (1.29 to 3.48, p=0.003)

*The lowest tertile with Lp-PLA_2 values <310 ng/mL was used as the reference group.

Model 1: univariate analysis
Model 2: adjusted for age, race and gender
Model 3: Model 2, plus adjustment for current smoking status, blood pressure, diabetes, HDL, LDL, CRP and Lp-PLA_2 - LDL interaction

A statistical interaction was found between Lp-PLA_2 and LDL. Therefore, it was appropriate to evaluate Lp-PLA_2 risk ratios in the high and low LDL subgroups. The median value of LDL for the cohort population was 130 mg/dL. This defined the high and low LDL subgroups. Tables 2a and 2b represent the univariate analysis of the risk ratios in the high and low LDL subgroups. The risk ratios were calculated from Cox regression employing the weighted case-cohort method with Barlow adjustment, n=1348.

Table 2a. Risk Ratios of CHD for Subjects with LDL <130 mg/dL

	$Lp\text{-}PLA_2$ Risk Ratio (95% CI)*		
$Lp\text{-}PLA_2$†	**Tertile 1**	**Tertile 2**	**Tertile 3**
Risk Ratio	**1.0**	**2.17** (1.41 to 3.36)	**3.52** (2.25 to 5.49)
#CHD cases/total subjects in category	51/215 **(23.7%)**	75/195 **(38.5%)**	77/163 **(47.2%)**

*The lowest tertile with $Lp\text{-}PLA_2$ values <310 ng/mL was used as the reference group.
†$Lp\text{-}PLA_2$ cutpoints based on the ARIC study population across all LDL levels.

Table 2b. Risk Ratios of CHD for Subjects with LDL $\geq$130 mg/dL

	$Lp\text{-}PLA_2$ Risk Ratio (95% CI)*		
$Lp\text{-}PLA_2$†	**Tertile 1**	**Tertile 2**	**Tertile 3**
Risk Ratio	**3.15** (2.08 to 4.77)	**3.66** (2.43 to 5.51)	**5.10** (3.43 to 7.57)
#CHD cases/total subjects in category	110/234 **(47.0%)**	126/247 **(51.0%)**	169/294 **(57.5%)**

*The lowest tertile for LDL <130 subgroup, with $Lp\text{-}PLA_2$ values <310 ng/mL, was used as the reference group.
†$Lp\text{-}PLA_2$ cutpoints based on the ARIC study population with LDL $\geq$130 mg/dL.

In the high LDL subgroup, the subgroup specific tertile groups yielded cutpoints of 350 and 460 ng/mL; the risk ratio increased with higher $Lp\text{-}PLA_2$ values. Therefore, for individuals with high LDL, a higher $Lp\text{-}PLA_2$ cutpoint should be considered. Further research is warranted to evaluate the $Lp\text{-}PLA_2$ - LDL interaction in the subgroup with high LDL. For the total population, $Lp\text{-}PLA_2$ was a significant predictor of risk for CHD for the high and intermediate groups versus the low $Lp\text{-}PLA_2$ (reference) group.

Ischemic Stroke

The levels of $Lp\text{-}PLA_2$ were evaluated in the ARIC study to determine its efficacy as a predictor of risk for stroke. A total of 223 stroke events were identified from the study group; of this, 194 (87%) were ischemic stroke associated with atherosclerosis, as classified by the ARIC investigators. A similar case-cohort study was designed, where samples from all the available ischemic stroke cases (194) were tested together with 762 appropriately matched participants without CHD or stroke at the time of censor (controls).

As with the study for CHD risk, Cox regression models were used to evaluate the association of $Lp\text{-}PLA_2$ and stroke in a univariate analysis (Model 1), a univariate analysis adjusted for demographics (Model 2), a multivariate model adjusted for demographics and other prognostic factors (Model 3), and all factors including CHD status (Model 4). The same tertile cutpoints (420 and 310 ng/mL, the 67th and 33rd percentiles, respectively) were applied to this study as for the earlier analyses. CHD status itself was found to be a predictor of risk, with a hazard ratio of 2.26 in a fully adjusted model. The hazard ratios of the Cox regression analyses demonstrated that $Lp\text{-}PLA_2$ was a significant and independent predictor of risk for ischemic stroke for the highest tertile, when compared to the lowest tertile of $Lp\text{-}PLA_2$, for all participants, with an increase of up to nearly 2-fold, even after adjustment for diabetes, lipids, blood pressure, smoking status, body mass index (BMI), other inflammatory markers and CHD status (see Table 3).

Table 3. Risk Ratios of Ischemic Stroke for All Subjects

	Lp-PLA$_2$ Risk Ratio (95% CI, p-value)*		
Lp-PLA$_2$	**Tertile 1**	**Tertile 2**	**Tertile 3**
# ischemic stroke cases/total subjects	47/283 (**16.6%)**	44/305 **(14.4%)**	103/368 **(28.0%)**
Model 1	**1.0**	**0.85** (0.57 to 1.29, p=0.45)	**1.79** (1.27 to 2.52, p=0.0010)
Model 2	**1.0**	**0.89** (0.59 to 1.35, p=0.58)	**2.09** (1.46 to 3.01, p=0.0001)
Model 3	**1.0**	**0.89** (0.58 to 1.36, p=0.59)	**1.81** (1.22 to 2.69, p=0.0034)
Model 4	**1.0**	**0.86** (0.56 to 1.31, p=0.48)	**1.75** (1.18 to 2.60, p=0.0057)

*The lowest tertile with Lp-PLA$_2$ values <310 ng/mL was used as the reference group.

Model 1: univariate analysis
Model 2: adjusted for age, race and gender
Model 3: Model 2, plus adjustment for diabetes, LDL, HDL, blood pressure, smoking, BMI and CRP
Model 4: Model 3, plus adjustment for CHD

Further analyses were performed to determine if Lp-PLA$_2$ was predictive of ischemic stroke across the complete range of systolic blood pressure (SBP) in the population, and to determine whether blood pressure and Lp-PLA$_2$ were additive in assessing risk for ischemic stroke. Systolic blood pressure tertile cutpoints were assigned by the 33rd and 67th percentiles of the population (113 and 130 mm Hg, respectively). The study population was divided into the low, mid and high range (1st, 2nd and 3rd tertile) of SBP and the low and high range of Lp-PLA$_2$ (below and above the median, 377 ng/mL in the ARIC study). The relative risk of each group was compared to the risk of events associated with the group in the 1st tertile of SBP and the group below the median of Lp-PLA$_2$ (Table 4).

Table 4. Risk Ratios of Ischemic Stroke: Additive Effects of Lp-PLA$_2$ and Systolic Blood Pressure

		Lp-PLA$_2$	
		Below Median	**Above Median**
SBP (mm Hg)	# ischemic stroke cases/total subjects in category	68/478 **(14.2%)**	126/478 **(26.4%)**
<113	29/270 **(10.7%)**	**1.00**	**2.29** (p=0.03)
113 to 130	60/337 **(17.8%)**	**2.05** (p=0.06)	**3.53** (p=0.0004)
>130	105/349 **(30.1%)**	**3.52** (p=0.0005)	**6.75** (p<0.0001)

The individuals above the median of Lp-PLA$_2$ concentration in the ARIC study and in the top tertile of systolic blood pressure (>130 mm Hg) had a risk ratio of 6.75 (p<0.0001), compared to those individuals below the median of Lp-PLA$_2$ and in the lowest tertile of blood pressure. These results indicated that Lp-PLA$_2$ and blood pressure were additive in their ability to predict risk, and that individuals in the highest groups of both variables were at the greatest risk of suffering an ischemic stroke associated with atherosclerosis.

PRODUCT SAFETY INFORMATION

Calibrator Set (1-6), Control Low and High	20X Wash Buffer	Stop Solution	Conjugate, TMB Reagent
[Xi] R36/38 S26/36/37/39	[Xi] R36/38 S26/36/37/39	[Xi] R34/41 S26/36/37/39	[Xi]

R34	Causes burns
R36	Irritating to eyes
R38	Irritating to skin
R41	Risk of serious danger to eyes
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice
S36/37/39	Wear suitable protective clothing, gloves, eye/face protection

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