AccuCardia™ ELISA Test System

REF ACC6301

IVD

CE

Rx Only

INTENDED USE

The ZEUS Scientific, Inc. AccuCardia™ ELISA Test System is an enzyme immunoassay for the *in vitro* quantitative determination of sPLA2-IIA (secreted phospholipase A2 – Group IIA) in human EDTA plasma. sPLA2-IIA results are to be used in conjunction with clinical evaluation and patient risk assessment as an aid in predicting risk of future myocardial infarction (MI) in patients with no prior history of cardiovascular events.

SIGNIFICANCE AND BACKGROUND

Cardiovascular disease (CVD) accounts for nearly half of the noncommunicable diseases worldwide, and is a leading cause of global death (1, 2). In 2008, the World Health Organization estimated that 17.3 million people died from CVD; the majority of these deaths being attributed to myocardial infarction (MI) and stroke (2 - 4). The severe clinical consequences of CVD, such as MI and stroke, are typically caused by atherosclerotic lesions; which ultimately disrupt the flow of blood to vital organs such as the heart and/or brain (5, 6). Close examination of these lesions reveal complex arterial structures consisting of connective tissue elements, inflammatory cells, lipids, and debris (5, 7). In recent years, research has shown that phosholipase A2 (PLA2) enzymes are key factors involved in atherosclerotic CVD; thus, they have emerged as promising biomarkers (8 - 11).

PLA2 enzymes are a family of proteins that catalyze the hydrolysis of phospholipids at the sn-2 position, yielding pro-inflammatory lysophospholipids and fatty acids (12 - 14). Secreted PLA2 (sPLA2) proteins make up a subgroup of this family, and consist of 10 calcium-dependent extracellular enzymes with relatively low molecular masses (8). A notable member of this subgroup, termed sPLA2-IIA, has been the focus of numerous basic research and clinical studies aimed at investigating its role in, and association with, cardiovascular conditions such as coronary artery disease (CAD) and atherosclerosis (8, 9). For example, several reports have linked increased plasma levels of sPLA2-IIA with recurrent events and adverse outcomes in patients with stable CAD (15, 16). Recently, additional studies have also shown that increased levels of sPLA2-IIA are predictive of recurrent cardiac events and death in patients presenting with acute coronary syndromes, such as MI and unstable angina (17, 18).

PRINCIPLE OF THE ASSAY

The AccuCardia test is a dual monoclonal antibody sandwich ELISA assay designed to quantitatively detect sPLA2-IIA protein in human plasma. A brief overview of the test procedure is as follows:

1. Test plasma, Calibrators, and Controls are diluted in Sample Diluent, then transferred to a microtiter plate containing immobilized anti-sPLA2-IIA antibody.
2. The diluted samples are incubated for one hour in the antibody-coated microwells. Sample-derived sPLA2-IIA is bound to the plate via interaction with the immobilized antibody. After incubation, the wells are washed to remove unbound plasma components.
3. A solution containing horseradish peroxidase (HRP)-conjugated anti-sPLA2-IIA antibody is then added to each well, and the plate is incubated again for one hour. After incubation, the plate is washed to remove unbound HRP conjugate.
4. The microwells containing immobilized sPLA2-IIA and HRP-conjugate are incubated for 10 minutes with peroxidase substrate solution, and hydrolysis of the substrate produces a color change.
5. After this incubation, the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution is directly proportional to the amount of sPLA2-IIA protein in the original test sample.

TEST SYSTEM COMPONENTS

Materials Provided:

Each Test System contains the following components in sufficient quantities to perform the number of tests indicated on the packaging label.

PLATE		1. Plate: 96 wells configured in twelve, 1 x 8-well, strips coated with a sPLA2-IIA specific antibody. The strips are packaged in a strip holder and sealed in an envelope with desiccant.
CONJ		2. Conjugate: sPLA2-IIA specific HRP-conjugated antibody. One, 15mL, white-capped, amber bottle. Ready to use (pH 7.5 ± 0.1).
CONTROL	I	3. Low Positive Control: One **green**-capped vial. Lyophilized.
CONTROL	II	4. Medium Positive Control: One **red**-capped vial. Lyophilized.
CAL	A	5. Calibrator A: One **white**-capped vial. Lyophilized.
CAL	B	6. Calibrator B: One **yellow**-capped vial. Lyophilized.
CAL	C	7. Calibrator C: One **orange**-capped vial. Lyophilized.
CAL	D	8. Calibrator D: One **blue**-capped vial. Lyophilized.
CAL	E	9. Calibrator E: One **clear**-capped vial. Lyophilized.
CAL	F	10. Calibrator F: One **purple**-capped vial. Lyophilized.
DIL	SPE	11. Sample Diluent: One, 30mL green-capped bottle. Ready to use (pH 7.5 ± 0.1).
SOLN	TMB	12. TMB: One, 15mL, amber-capped, amber bottle containing 3, 3', 5, 5' - tetramethylbenzidine (TMB). Ready to use.
SOLN	STOP	13. Stop Solution: One, 15mL, red-capped, bottle containing 1M H_2SO_4, 0.7M HCl. Ready to use.
WASHBUF	10X	14. Wash Buffer Concentrate (10X): One, 100mL, clear-capped, bottle containing a 10X concentrated phosphate-buffered-saline and Tween-20 solution (clear solution). Preparation: Dilute 1 part concentrate + 9 parts deionized or distilled water. **NOTE: 1X solution will have a pH of 7.2 ± 0.2.**

NOTES:

1. The Stop Solution and Wash Buffer are not test system/lot number dependent and may be used interchangeably with any of the ZEUS ELISA™ Test Systems.
2. Test System also contains:
   a. Component Label containing lot specific information for the materials packaged inside the Test System box.
   b. Package Insert providing instructions for use.

PRECAUTIONS

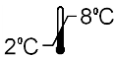
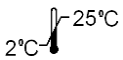
1. For *in vitro* diagnostic use.
2. Follow normal precautions handling laboratory reagents. In case of contact with eyes, rinse immediately with copious amounts of water and seek medical advice. Wear suitable protective clothing, gloves, and eye/face protection. Do not breathe vapor. Dispose of waste observing all local, state, and federal laws.
3. The wells of the ELISA plate do not contain viable organisms. However, consider the strips **potentially biohazardous materials** and handle accordingly.

4. The Controls are **potentially biohazardous materials**. Source materials from which these products were derived were found negative for HIV-1 antigen, HbsAg and for antibodies against HCV and HIV by approved test methods. However, since no test method can offer complete assurance that infectious agents are absent, handle these products at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories": Current Edition; and OSHA's Standard for Bloodborne Pathogens (19).
5. Adherence to the specified time and temperature of incubations is essential for accurate results. **All reagents must be allowed to reach room temperature (20 – 25°C) before starting the assay**. Return unused reagents to refrigerated temperature immediately after use.
6. Improper washing could cause false positive or false negative results. Be sure to minimize the amount of any residual wash solution; (e.g., by blotting or aspiration) before adding Conjugate or Substrate. Do not allow the wells to dry out between incubations.
7. The Stop Solution is TOXIC if inhaled, has contact with skin or is swallowed. It can cause burns. In case of accident or feeling ill, seek medical advice immediately.
8. The TMB Solution is HARMFUL. It is irritating to eyes, the respiratory system and skin.
9. The Wash Buffer concentrate is an IRRITANT. It is irritating to eyes, the respiratory system and skin.
10. Wipe the bottom of the plate free of residual liquid and/or fingerprints that can alter optical density (OD) readings.
11. Dilution or adulteration of these reagents may generate erroneous results.
12. Do not use reagents from other sources or manufacturers.
13. TMB Solution should be colorless, very pale yellow, very pale green, or very pale blue when used. Contamination of the TMB with Conjugate or other oxidants will cause the solution to change color prematurely. Do not use the TMB if it is noticeably blue in color.
14. Never pipette by mouth. Avoid contact of reagents and patient specimens with skin and mucous membranes.
15. Avoid microbial contamination of reagents. Incorrect results may occur.
16. Cross contamination of reagents and/or samples could cause erroneous results.
17. Reusable glassware must be washed and thoroughly rinsed free of all detergents.
18. Avoid splashing or generation of aerosols.
19. Do not expose reagents to strong light during storage or incubation.
20. Allowing the microwell strips and holder to equilibrate to room temperature prior to opening the protective envelope will protect the wells from condensation.
21. Collect the wash solution in a disposal basin. Treat the waste solution with disinfectant (i.e.: 10% household bleach – 0.5% Sodium Hypochlorite). Avoid exposure of reagents to bleach fumes.
22. Caution: Neutralize any liquid waste at an acidic pH before adding to a bleach solution.
23. Do not use ELISA plate if the indicator strip on the desiccant pouch has turned from blue to pink.
24. Do not allow the Conjugate to come in contact with containers or instruments that may have previously contained a solution utilizing Sodium Azide as a preservative. Residual amounts of Sodium Azide may destroy the Conjugate's enzymatic activity.
25. Do not expose any of the reactive reagents to bleach-containing solutions or to any strong odors from bleach-containing solutions. Trace amounts of bleach (sodium hypochlorite) may destroy the biological activity of many of the reactive reagents within this Test System.

MATERIALS REQUIRED BUT NOT PROVIDED

1. ELISA microwell reader capable of reading at a wavelength of 450nm.
2. Software capable of computing results using a 4-parameter logistic curve-fitting equation
3. Incubator capable of 37°C incubation.
4. Sample dilution plate
5. Pipettes capable of accurately delivering 10 – 200µL.
6. Multichannel pipette capable of accurately delivering 50 – 200µL.
7. Reagent reservoirs for multichannel pipettes.
8. Microwell washing system.
9. Distilled or deionized water.
10. One liter graduated cylinder.
11. Serological pipettes.
12. Disposable pipette tips.
13. Paper towels.
14. Laboratory timer to monitor incubation steps.
15. Disposal basin and disinfectant (i.e.: 10% household bleach – 0.5% Sodium Hypochlorite).

STORAGE CONDITIONS

	Coated Microwell Strips: Immediately reseal extra strips with desiccant and return to proper storage. After opening – strips are stable for 30 days, as long as the indicator strips on the desiccant pouch remains blue.
	Reconstituted Calibrators/Controls are stable for up to 30 days.
	Conjugate – DO NOT FREEZE.
	Unopened Test System, Calibrators, Positive Controls, TMB, Sample Diluent
	Stop Solution: 2 – 25°C Wash Buffer (1X): 20 – 25°C for up to 7 days, 2 – 8°C for 30 days. Wash Buffer (10X): 2 – 25°C

SPECIMEN COLLECTION

1. ZEUS Scientific recommends that the user carry out specimen collection using standard of practice phlebotomy techniques and in accordance with CLSI document M29: Protection of Laboratory Workers from Infectious Disease (Current Edition).
2. No known test method can offer complete assurance that human blood samples will not transmit infection. Therefore, consider all blood derivatives potentially infectious.
3. Use only **EDTA plasma** obtained by approved aseptic venipuncture procedures (21, 22). Avoid using hemolyzed, lipemic, or bacterially contaminated plasma.
4. While CLSI has published recommended guidelines for sample storage (20), internal real-time sample stability studies have demonstrated that endogenous sPLA2-IIA is stable for up to 14 days in EDTA plasma when stored at 2 - 8°C, and for up to 7 days when stored at room temperature. An internal freeze/thaw study has also demonstrated that endogenous sPLA2-IIA in EDTA plasma is stable for at least 5 freeze/thaw cycles.

ASSAY PROCEDURE

1. **Setup:**
   a. Remove the individual components from storage and allow them to warm to room temperature (20 - 25°C) for approximately 30 minutes.
   b. If using the kit for the first time, reconstitute the lyophilized Calibrators and Controls as follows:
      i. Add 250µL of distilled water to each vial reconstituting from lowest to highest concentration and using a fresh disposable pipette tip each time.
      ii. Allow the freshly reconstituted vials to sit for 10 - 15 minutes at room temperature.
      iii. Vortex before use making certain there is no undissolved lyophilized material on the underside of the cap.
      NOTE: Once reconstituted, the Calibrators and Controls are stable for 30 days.

c. Prepare 1X Wash Buffer by adding the contents of the 10X Wash Buffer bottle to 900mL of deionized water. Mix by gentle inversion.

d. Determine the number of microwells needed. For each run, wells must be assigned for duplicate analysis of six Calibrators, Control I and Control II. Assign additional wells for duplicate analysis of patient samples. Check software and reader requirements for the correct Calibrator/Control configurations. Return unused strips to the resealable pouch with desiccant, seal, and return to storage between 2 - 8°C.

EXAMPLE PLATE SET-UP						
	1	2	3	4	5	6
A	Calibrator A	Calibrator A	Patient Specimen 1	Patient Specimen 1	Patient Specimen 9	Patient Specimen 9
B	Calibrator B	Calibrator B	Patient Specimen 2	Patient Specimen 2	Etc.	
C	Calibrator C	Calibrator C	Patient Specimen 3	Patient Specimen 3		
D	Calibrator D	Calibrator D	Patient Specimen 4	Patient Specimen 4		
E	Calibrator E	Calibrator E	Patient Specimen 5	Patient Specimen 5		
F	Calibrator F	Calibrator F	Patient Specimen 6	Patient Specimen 6		
G	Control I	Control I	Patient Specimen 7	Patient Specimen 7		
H	Control II	Control II	Patient Specimen 8	Patient Specimen 8		

2. **Procedure:**

a. Dilute each Calibrator, Control, and patient specimen 1:11 in Sample Diluent (i.e.: 25µL sample + 250µL Sample Diluent).

b. To individual wells, add 100µL of each diluted Calibrator, Control, and patient specimen in duplicate according to the ELISA plate setup map shown above. (Note: Ensure the samples are properly mixed, and use a different pipette tip for each sample)

c. Incubate the plate **uncovered** at 37 ± 1°C for 60 ± 5 minutes. **NOTE: Do NOT seal the plate during incubation.**

d. Wash the microwell strips five times. For automated washing, set the dispensing volume to 350µL/well. Set the wash cycle for five washes with no delay between washes. If necessary, the microwell plate may be removed from the washer, inverted over a paper towel and tapped firmly to remove any residual wash solution from the microwells.

e. Add 100µL of the Conjugate to each well at the same rate and order as the specimens.

f. Incubate the plate **uncovered** at 37 ± 1°C for 60 ± 5 minutes. **NOTE: Do NOT seal the plate during incubation.**

g. Wash the microwell strips according to Step d. above in this procedure.

h. Add 100µL of TMB to each well at the same rate and order as the specimens.

i. Incubate the plate **uncovered** at room temperature (20 - 25°C) for 10 minutes ± 1 minute.

j. Stop the reaction by adding 100µL of Stop Solution to each well at the same rate and order as the TMB. Positive samples will turn from blue to yellow.

k. Set the microwell plate reader to read at a wavelength of 450nm and measure the optical density (OD) of each well. Read the plate within 30 minutes of the addition of the Stop Solution. Be sure the bottom of the plate has been wiped clean and dry to prevent interference with the OD reading.

ABBREVIATED TEST PROCEDURE

1. Reconstitute Calibrators and Controls .
2. Dilute Calibrators, Controls and samples 1:11 in Sample Diluent
3. Add diluted Calibrators, Controls, and samples to duplicate microwells - 100µL/well.
4. → *Incubate uncovered 60 ± 5 minutes at 37° ± 1°C.*
5. Wash five times, invert on paper towel to remove residual liquid.
6. Add Conjugate - 100µL/well.
7. → *Incubate uncovered 60 ± 5 minutes at 37° ± 1°C.*
8. Wash five times, invert on paper towel to remove residual liquid.
9. Add TMB - 100µL/well.
10. → *Incubate uncovered 10 ± 1 minute at 20 - 25°C.*
11. Add Stop Solution - 100µL/well.
12. Read optical density at 450 nm within 30 minutes.

QUALITY CONTROL

1. Each time the assay is performed, the Calibrators, Controls, and samples must be run in duplicate.
2. Additional control material may be tested per guidelines or requirements of local, state, and/or federal regulations or accrediting organizations.
3. Refer to NCCLS document C24: Statistical Quality Control for Quantitative Measurements for guidance on appropriate QC practices.
4. The Low and Moderate Positive Controls are intended to monitor for substantial reagent failure. The Controls should yield ng/mL values within the lot-specific ranges listed on the component label provided with each kit.
5. If either, or both, of the Controls fall outside the ranges listed on the lot-specific label, assay results may be invalid, and testing **must** be repeated.

INTERPRETATION OF RESULTS

1. Enter the lot-specific ng/mL value for each Calibrator into the appropriate section of the desired 4-parameter logistic curve fitting software. The ng/mL values for each Calibrator are listed on the component label provided with each kit. **NOTE: Be sure to program the software to calculate the mean ng/mL value using the OD values derived from duplicate analysis of each Calibrator, Control, and patient sample.**
2. Test results should be interpreted in conjunction with the patient's clinical evaluation and, potentially, the results of other diagnostic procedures.
3. Cardiac risk analysis calculations have been performed on clinical samples using a 25ng/mL sPLA2-IIA EDTA plasma concentration as the cutpoint for increased risk based on information gathered during a pilot study previously performed by ZEUS.
4. **Clincal Results Reporting**: For measured results: a.) 0 up to 20ng/mL, report as <20ng/mL; b.) 20 - 200ng/mL, report as measured value; c.) >200ng/mL should be reported as >200 ng/mL. **In addition to the numerical result, an interpretive comment must also be reported.** For sPLA2-IIA EDTA plasma results <25ng/mL, report with the interpretive comment "Results of <25ng/mL are statistically normal and therefore such individuals are not representative of being at risk of a future MI". Results ≥25ng/mL should be reported with the interpretive comment "Risk of myocardial infarction within one year may be 2 - 3 times higher than it would be if sPLA2-IIA was <25ng/mL".
5. If desired, specimens with results exceeding 200ng/mL may be repeat-tested after serial dilution in Sample Diluent. Once the diluted sample yields two consecutive values within the quantitative range of the assay, the dilution factor can be used to back-calculate the actual value as follows: **Example:** 4p software assigns a ">525 ng/mL" result to a patient sample. To begin retesting, the plasma sample is serially diluted (1:2, 1:4, 1:8, 1:16, etc…) in Sample Diluent, and each dilution is tested in duplicate according to the standard procedure. The results after serial dilution and retesting are as follows: 1:2 = ">525ng/mL", 1:4 = 350ng/mL, 1:8 = 175ng/mL. 1:16 = 87.5ng/mL. **Calculations:** Measured value x Dilution factor = Actual ng/mL, so 175 x 8 = 1400ng/mL or 87.5 x 16 = 1400ng/mL.

LIMITATIONS OF THE ASSAY

1. Only **EDTA plasma** samples should be tested with this assay. Lithium heparin plasma has been demonstrated to be **incompatible** with the assay. Serum, and plasma derived from blood collection tubes containing other anticoagulants, have not been fully tested/validated, and thus should be avoided.
2. Whole blood is not an appropriate sample type for this assay.

PERFORMANCE CHARACTERISTICS

1. **Limits of Blank, Detection, and Quantitation**

a. **Limit of Blank (LOB) for Calibrator Diluent = 7.03ng/mL**

Two technicians each analyzed 60 replicates of calibrator diluent, totaling 120 replicates.

i. Average ng/mL from two independent runs (n = 120 data points) = 3.99ng/mL
ii. Standard deviation from two independent runs (n = 120 data points) = 1.85ng/mL
iii. LOB = Avg + 1.645(StdDev) = 3.99 + 1.645(1.85) = 7.03ng/mL

b. **Limit of Blank (LOB) for EDTA plasma = 7.15ng/mL**

Five different units of EDTA plasma (deficient for sPLA2-IIA) were assayed by two technicians on five different days. On each day, 12 assay replicates were performed for each plasma unit, totaling 300 replicates.

i. Average ng/mL from five independent runs (n = 300 data points) = 4.55ng/mL
ii. Standard deviation from five independent runs (n = 300 data points) = 1.58ng/mL
iii. LOB = Avg + 1.645(StdDev) = 4.55 + 1.645(1.58) = 7.1ng/mL

c. **Limit of Detection (LOD) = 10ng/mL**

The LOD for the assay was determined using EDTA plasma containing both recombinant and endogenous sPLA2-IIA at various levels, and was considered valid if ≥95% of the replicates tested at a given level yielded values greater than the established LOB of 7.15ng/mL. For each level of sPLA2-IIA, a minimum of 50 replicates were assayed by two technicians.

d. **Lower Limit of Quantitation (LLOQ) = 20ng/mL**

The LLOQ was determined based upon defined precision criteria (i.e. CV values of 20% or less) for samples containing a given level of recombinant and/or endogenous sPLA2-IIA. Also considered was the in-house and multi-site precision study performance data.

e. **Upper Limit of Quantitation (ULOQ) = 200ng/mL**

Selection of the ULOQ was based upon defined precision criteria (i.e. CV values of 20% or less) for samples containing a given level of recombinant and/or endogenous sPLA2-IIA. Taken into consideration were the in-house and multi-site precision study performance, as well as the Linearity and Dilutional Recovery studies.

2. **Assay Detection Ranges**

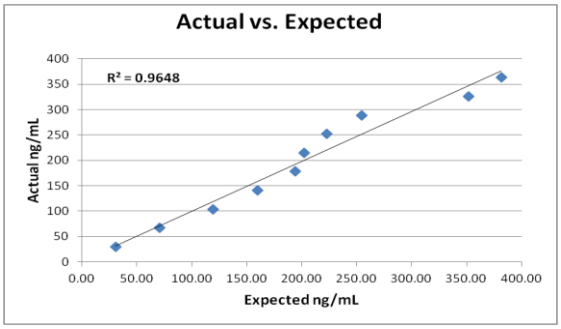
a. **Analytical Range = 10 - 500*ng/mL** (* is the value of the highest Calibrator in the reagent set being used.)
b. **Quantitative = 20 - 200ng/mL NOTE: Clinical cut-point for increased risk is 25ng/mL, the value of the lowest non-zero calibrator.**

3. **Linearity and Dilutional Recovery**

a. **Linearity:** An EDTA plasma sample spiked with high levels of sPLA2-IIA was diluted with an EDTA plasma pool containing low levels of endogenous sPLA2-IIA, resulting in multiple concentration levels. The expected results were compared to the actual results, and the outcomes are shown below.

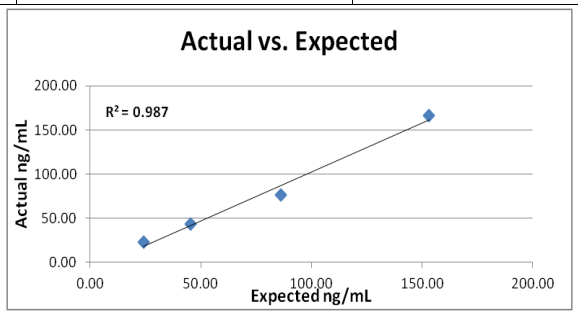
Sample	Expected Value (ng/mL)	Measured Value (Mean ng/mL)	StdDev	% CV	% Recovery
Pool 1	30	30.64	0.64	2.09	102.12
Pool 2	67	70.37	6.16	8.76	105.03
Pool 3	104	119.42	4.24	3.55	114.83
Pool 4	141	160.05	14.02	8.76	113.51
Pool 5	178	194.01	8.22	4.24	109.00
Pool 6	215	202.33	20.90	10.33	94.11
Pool 7	252	222.88	19.30	8.66	88.44
Pool 8	289	254.73	19.69	7.73	88.14
Pool 9	326	351.93	48.11	13.67	107.95
Pool 10	363	381.54	26.16	6.86	105.11
Pool 11	400	* ND	* ND	* ND	* ND

* ND = None Determined due to three of the replicates being designated as 'out of range' (>525ng/mL) by 4p software.



b. **Dilutional Recovery:** An EDTA plasma sample with high levels of endogenous sPLA2-IIA was serially diluted in the sample diluent. Diluted samples were assayed in duplicate, and resultant data were analyzed for agreement and % recovery for expected values and actual values throughout the dilution series.

Dilution Factor	Mean ng/mL	Expected ng/mL	% Recovery
Neat	332.40	NA	NA
1:2	153.21	166.20	92
1:4	86.22	76.61	113
1:8	45.38	43.11	105
1:16	24.05	22.69	106
1:32	13.15	12.02	109
1:64	6.69	6.58	102
1:128	3.13	3.35	94



4. **Precision**

a. **In-house Precision Study:** Plasma samples containing endogenous sPLA2-IIA at various levels were assayed in duplicate, twice per day, for 20 days by a single technician, using a single lot of kits.

Sample	Mean	Repeatability		Inter-Run		Inter-Day		Total	
		Std Dev	% CV	Std Dev	% CV	Std Dev	% CV	Std Dev	% CV
1	5.31	1.687	31.8	0	0	1.647	31.0	2.357	44.4
2	19.73	1.468	7.4	0	0	1.147	5.8	1.863	9.4
3	38.09	2.250	5.9	0	0	1.769	4.6	2.862	7.5
4	70.98	4.272	6.0	3.407	4.8	2.451	3.5	5.989	8.4
5	138.45	7.403	5.3	1.684	1.2	4.627	3.3	8.891	6.4
6	183.93	13.768	7.5	4.366	2.4	5.131	2.8	15.328	8.3

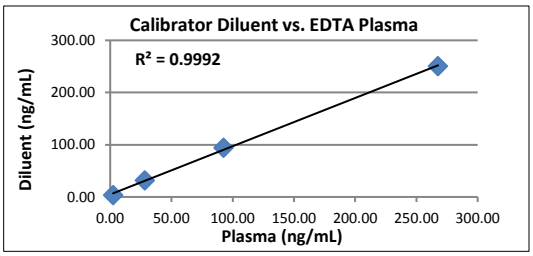
NOTE: A standard deviation of zero is obtained when the mean square error for that factor is larger than the mean square for the factor.

b. **Multi-site precision study:** Plasma samples containing endogenous sPLA2-IIA at various levels were assayed at three independent testing sites. At each site, two technicians assayed the precision samples in triplicate, twice per day, for five days.

Sample	Site	Lot	N	Mean (ng/mL)	Repeatability		Inter-Day		Inter-Run		Inter-Operator		Total	
					Std Dev	% CV	Std Dev	% CV	Std Dev	% CV	Std Dev	% CV	Std Dev	% CV
1	1	020	60	7.551	5.467	72.403	1.470	19.468	0.777	10.291	0.000	0.000	5.715	75.678
		021	60	3.612	3.576	99.009	1.450	40.157	0.000	0.000	1.535	42.502	4.153	114.985
		022	60	4.610	5.449	118.200	0.000	0.000	0.000	0.000	0.000	0.000	5.449	118.200
	2	020	60	7.356	3.690	50.160	2.535	34.458	2.820	38.338	2.902	39.444	6.034	82.030
		021	60	5.294	1.887	35.652	1.336	25.230	1.010	19.081	0.102	1.931	2.525	47.702
		022	60	5.076	1.779	35.046	1.552	30.575	0.000	0.000	0.692	13.626	2.460	48.463
	3	020	60	9.438	2.730	28.929	0.728	7.717	1.361	14.423	1.616	17.125	3.529	37.386
		021	60	4.506	3.097	68.733	1.543	34.253	0.314	6.965	0.915	20.318	3.593	79.742
		022	60	5.776	2.003	34.688	1.908	33.029	0.000	0.000	1.153	19.955	2.997	51.888
2	1	020	60	12.775	2.347	18.370	1.216	9.522	0.331	2.589	0.778	6.087	3.408	26.677
		021	60	11.285	2.595	22.992	1.077	9.544	2.035	18.034	2.440	21.622	4.241	37.583
		022	60	10.072	1.693	16.806	1.372	13.620	0.648	6.434	1.618	16.064	2.790	27.702
	2	020	60	14.755	2.347	15.904	1.288	8.731	0.382	2.589	0.161	1.089	2.709	18.359
		021	60	12.201	4.163	34.118	0.434	3.558	0.108	0.883	0.000	0.000	1.787	14.649
		022	60	12.227	1.156	9.451	0.583	4.768	0.441	3.603	0.741	6.061	1.555	12.719
	3	020	60	17.198	1.169	6.795	0.340	1.976	0.000	0.000	2.206	12.826	2.519	14.649
		021	60	11.873	3.153	26.558	1.131	9.529	0.547	4.606	0.000	0.000	4.462	37.583
		022	60	12.455	2.475	19.870	2.504	20.102	0.483	3.878	0.000	0.000	3.553	28.530
3	1	020	60	18.461	3.263	17.677	1.490	8.071	0.000	0.000	0.878	4.756	3.693	20.006
		021	60	17.688	2.737	15.474	0.000	0.000	1.182	6.683	0.425	2.402	3.012	17.026
		022	60	16.380	1.705	10.411	0.941	5.747	0.000	0.000	0.899	5.488	2.145	13.097
	2	020	60	20.564	1.404	6.827	0.538	2.617	0.741	3.606	0.000	0.000	1.676	8.152
		021	60	17.787	1.387	7.799	0.677	3.807	0.234	1.313	0.072	0.403	1.563	8.787
		022	60	17.820	1.257	7.054	0.643	3.611	0.778	4.368	1.105	6.199	1.955	10.968
	3	020	60	23.561	2.692	11.425	0.543	2.304	0.000	0.000	2.829	12.008	3.943	16.734
		021	60	18.562	2.764	14.890	1.291	6.955	0.000	0.000	0.000	0.000	3.050	16.434
		022	60	18.686	2.128	11.388	1.878	10.048	0.000	0.000	1.550	8.297	3.234	17.306
4	1	020	60	60.477	3.831	6.335	2.161	3.573	1.369	2.264	1.407	2.326	4.817	7.964
		021	60	60.236	5.322	8.835	1.374	2.281	2.998	4.977	3.605	5.984	7.224	11.993
		022	60	57.450	3.898	6.785	2.693	4.688	3.491	6.077	0.679	1.183	5.924	10.312
	2	020	60	59.860	3.967	6.627	1.942	3.244	0.734	1.227	0.000	0.000	4.477	7.480
		021	60	55.522	2.927	5.272	1.266	2.281	0.000	0.000	0.000	0.000	3.339	6.013
		022	60	54.829	3.844	7.012	1.411	2.573	0.000	0.000	0.000	0.000	4.095	7.469
	3	020	60	71.786	5.467	7.616	2.285	3.183	0.000	0.000	8.770	12.217	10.584	14.744
		021	60	60.977	4.867	7.982	3.882	6.367	3.035	4.977	4.188	6.868	7.503	12.305
		022	60	62.795	5.516	8.785	2.049	3.262	0.000	0.000	9.464	15.072	11.144	17.747
5	1	020	60	123.704	12.533	10.131	3.076	2.487	3.003	2.428	8.663	7.003	15.830	12.797
		021	60	126.800	10.757	8.483	6.015	4.744	5.653	4.458	5.128	4.045	14.497	11.433
		022	60	122.244	10.413	8.518	0.906	0.741	6.289	5.144	3.499	2.863	12.690	10.381
	2	020	60	115.855	7.788	6.723	0.000	0.000	1.920	1.657	0.000	0.000	8.022	6.924
		021	60	113.414	5.663	4.993	1.587	1.399	0.000	0.000	3.164	2.790	6.678	5.889
		022	60	111.082	7.747	6.974	1.589	1.431	1.529	1.376	3.428	3.086	8.754	7.880
	3	020	60	146.994	10.811	7.355	8.177	5.563	0.000	0.000	17.483	11.894	22.123	15.050
		021	60	126.340	9.102	7.205	6.587	5.214	2.371	1.877	7.845	6.210	13.907	11.008
		022	60	131.863	10.902	8.267	7.273	5.516	1.355	1.027	16.087	12.200	20.793	15.769
6	1	020	60	284.508	61.785	21.716	13.305	4.677	0.000	0.000	12.645	4.445	64.454	22.654
		021	60	327.225	67.163	20.525	42.503	12.989	50.952	15.571	2.347	0.717	94.412	28.852
		022	60	324.139	79.068	24.393	21.682	6.689	6.777	2.091	0.000	0.000	82.267	25.380
	2	020	60	245.218	40.572	16.545	12.619	5.146	7.322	2.986	11.546	4.709	44.634	18.202
		021	60	241.972	19.655	8.123	8.714	3.601	2.461	1.017	1.736	0.717	21.710	8.972
		022	60	243.891	28.356	11.627	14.456	5.927	9.538	3.911	9.916	4.066	34.675	14.217
	3	020	60	295.726	30.463	10.301	19.536	6.606	6.444	2.179	8.914	3.014	37.824	12.790
		021	60	274.500	39.767	14.487	9.221	3.359	24.837	9.048	12.329	4.492	49.349	17.978
		022	60	278.645	25.025	8.981	8.575	3.077	4.558	1.636	12.634	4.534	29.667	10.647

5. **Calibrator Diluent vs. EDTA plasma**

Recombinant sPLA2-IIA was spiked into Calibrator Diluent, and 3 different EDTA plasma units, at target concentrations of approximately 30, 100, and 300ng/mL. Non-spiked and spiked plasma/diluent were assayed in triplicate, and the agreement between ng/mL values were evaluated for respective matrices. As shown below, the ng/mL values obtained from Calibrator Diluent and EDTA plasma are in good agreement ($R^2 = 0.9992$).



sPLA2-IIA Level	Calibrator Diluent Mean ng/mL	EDTA Plasma Mean ng/mL (n=3 donors)
0	2	4
Low	28	32
Medium	93	94
High	268	251

6. **Hook Effect**

Recombinant sPLA2-IIA was spiked into Calibrator Diluent to a concentration of 5000ng/mL, then serially diluted to near the LOD of 10ng/mL. Diluted samples were assayed in triplicate. As shown below, no false negatives were observed, demonstrating that the assay is not susceptable to a 'hook effect' up to at least 5000ng/mL of sPLA2-IIA.

Targeted Spike (ng/mL)	Replicate A (ng/mL)	Replicate B (ng/mL)	Replicate C (ng/mL)	Mean Measured (ng/mL)	Expected (ng/mL)	% Recovery
5,000	>525.000	>525.000	>525.000	NA	NA	NA
2,500	>525.000	>525.000	>525.000	NA	NA	NA
1,250	>525.000	>525.000	>525.000	NA	NA	NA
625	>525.000	>525.000	>525.000	NA	NA	NA
312.5	366	327	311	334.68	NA	NA
156.25	183	171	158	170.79	167.34	102.06
78.13	86	90	81	85.60	85.40	100.24
39.06	44	45	44	44.29	42.80	103.49
19.53	20	21	20	20.17	22.15	91.09
9.76	9	10	9	9.42	10.09	93.44
0	2	2	2	2.18	NA	NA

7. **Interfering Substances and Cross Reactivity**

a. **Non-immunologic Substances:** The analysis was performed on normal human plasma spiked with various relevant endogenous and exogenous substances. For each substance/concentration, testing was performed using 0, 50, and 150ng/mL of sPLA2-IIA. The respective test concentrations of the various substances are listed below:

Substance	High (mg/mL)	Low (mg/mL)
Albumin (Human)	50	35
Bilirubin	0.15	0.01
Cholesterol	2.5	1.5
*Hemolysate	5	0.5
Intralipids	7.5	3
Triglycerides	5	1.5
Acetylsalicylic Acid	0.652	0.252
Acetaminophen	0.2	0.0197
L-ascorbic Acid	0.0662	0.0132
Atorvastatin	0.22	0.022
Tolbutamide	0.64	0.064

*** Hemolysate was prepared and tested according to Appendix G of CLSI document EP07.**

Outcome: None of the above-listed substances exhibited significant positive or negative interference on assay results (i.e. all yielded between 80 - 120% signal relative to the respective matrix control values) at both the high and low levels of spiked sPLA2-IIA.

b. **Immunologic Substances:** Testing was also performed with HAMA/Rf-spiked plasma samples, and no significant interference was observed.

c. **Cross Reactivity:** The following recombinant proteins, representing various human non-IIA sPLA2 isoforms, as well as mouse orthologs, were spiked into assay-specific sample diluent to a final concentration of 10, 100, 500, and 1000 ng/mL and were assayed. None of the proteins exhibited cross reactivity at any of the levels tested.

- sPLA2 hGV
- sPLA2 hGX
- sPLA2 hGIID
- sPLA2 hGIIF
- sPLA2 hGIII
- sPLA2 mGIIA
- sPLA2 mGX
- sPLA2 mGV
- sPLA2 mGIID
- sPLA2 mGIIF
- sPLA2 mGIB

NOTE: 'h' prefix denotes human amino acid sequence and 'm' prefix denotes mouse amino acid sequence.

8. **Clinical Study**

Plasma drawn at entry from 6500 subjects enrolled in the REasons for the Geographic And Racial Differences in Stroke (REGARDS) Study was assayed for sPLA2-IIA at a single CLIA certified laboratory using the AccuCardia™ ELISA Test System. Longitudinal health history over many years was examined for each participant.

a. **ZEUS REGARDS Study Demographics:**

i. **Race/Gender Statistics:** A total of 6500 Subjects (3183 females/3317 males) participated. The racial/gender makeup is shown in Table 1 below:

Table 1: Racial/Gender Statistics

Race/Gender	Number of Subjects	% of Study Total
African-American Female	1460	22.5
African-American Male	1058	16.3
Caucasian Female	1723	26.5
Caucasian Male	2259	34.8
Totals	6500	100.0

ii. **Race/Gender/Age Statistics:** Shown in Table 2 below:

Table 2: Sample Statistics for Age in Years at Entry by Gender and Race

Gender			Race	
			Caucasian	African-American
Female	N		1723	1460
	Mean		66.08	64.79
	Standard Deviation		9.752	9.323
	Range		47	47
	Minimum		45	45
	Maximum		92	92
	Percentiles	25	59.00	58.00
		50	66.00	65.00
		75	74.00	71.00

Table 2: Sample Statistics for Age in Years at Entry by Gender and Race (continued)

Male	N		2259	1058
	Mean		67.86	65.46
	Standard Deviation		9.163	8.821
	Range		48	45
	Minimum		45	45
	Maximum		93	90
	Percentiles	25	61.00	59.00
		50	68.00	65.00
		75	75.00	72.00

b. **ZEUS REGARDS Study Findings:**

Nine hundred and thirty-one (931) of the 6500 patients experienced a Myocardial Infarction (MI) during the span of the study. For those individuals who suffered an MI, the average time to the MI from the date the blood was collected was 3.3 years with a median of 3.0 years. Referring to Chart 1, a signficant difference in the time to a cardiac event (MI) between Subjects whose study entry sPLA2-IIA results were <25ng/mL and those whose results were ≥25ng/mL is clearly demonstrated. These results indicate that Subjects with values of sPLA2-IIA ≥25ng/mL at Study entry are two to three times more likely to experience an MI over the course of the REGARDS Study than Subjects whose sPLA2-IIA values are <25ng/mL. A Cox regression model also indicates the increased risk for current smokers over non-smokers and past smokers over non-smokers, males over females, and Subjects older than 66 years of age over Subjects less than 66 years of age.

Data were also analyzed to determine whether there was an increased risk for a future cardiac event (MI) within the year following the blood sample collection for those Subjects with an elevated level of sPLA2-IIA (≥25ng/mL) at the time of study entry and to quantify that risk if one was found. Statistical analyses on the data generated in the ZEUS REGARDS Study indicated that age, gender, and smoking status were all significant predictors of risk for an MI during the first year. This is not surprising since these risk factors have been well documented in multiple studies for decades and were risk factors found when data over the course of the REGARDS followup was examined. Interestingly, race was not found to be a signficant risk factor for MI over the course of the first year. Because of the known contribution of age, gender and smoking as risk factors for an MI, incidence ratios were calculated to determine whether sPLA2-IIA values ≥25ng/mL were also a signficant risk factor for MI during the first year after sample collection/analysis. The conclusion from the analysis was that a plasma level of sPLA2-IIA ≥25ng/mL is a risk factor for the development of a myocardial infarction within one year of the assay determination. See Table 3. **NOTE: P_r <0.05 indicates significant contribution to risk**. Smoking appears to be such a strong contributor to risk it tends to overwhelm the calculations as seen in Table 3 for females <66 years old for past and current smokers, for females >66 years old for past smokers, and for male past smokers >66 years old. Three cohorts in the analysis could not be evaluated only because there were no specific events in that time interval for that cohort.

Chart 1: Time to MI Based on sPLA2-IIA Results

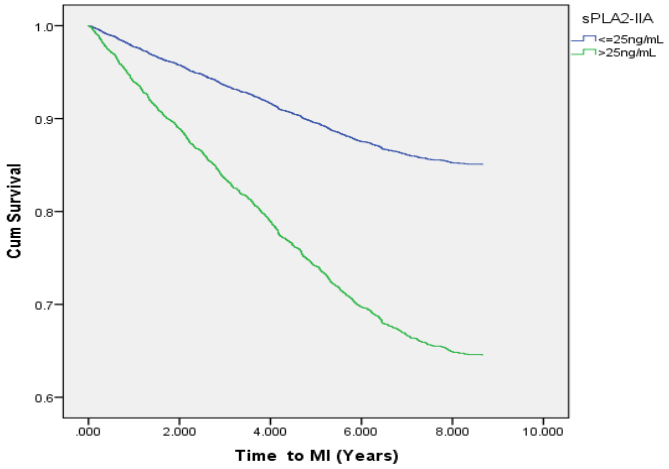


Table 3: Analysis Results - Myocardial Infarction

Gender	Age	Smoking Status	MI	Level of sPLA2-IIA		IDR	p	Pr	sPLA2-IIA ≥25 Significant Contribution
				≥25ng/mL	<25ng/mL				
Female	<66	Never	Events (within 1 Year)	2	3	14.56	0.044	0.02	Yes
			Population Time	35.5	775.5				
Female	<66	Past	Events (within 1 Year)	2	9	2.80	0.073	0.19	No
			Population Time	34.8	438.5				
Female	<66	Current	Events (within 1 Year)	1	8	1.32	0.086	0.55	No
			Population Time	15.1	159.6				
Female	>66	Never	Events (within 1 Year)	0	3	Can Not Determine			
			Population Time	14.0	405.6				
Female	>66	Past	Events (within 1 Year)	3	14	3.23	0.062	0.09	No
			Population Time	33.9	511.8				
Female	>66	Current	Events (within 1 Year)	0	6	Can Not Determine			
			Population Time	8.4	150.8				
Male	<66	Never	Events (within 1 Year)	1	4	39.40	0.006	0.031	Yes
			Population Time	3.0	467.5				
Male	<66	Past	Events (within 1 Year)	2	15	10.25	0.013	0.02	Yes
			Population Time	8.6	661.8				
Male	<66	Current	Events (within 1 Year)	0	17	Can Not Determine			
			Population Time	13.0	308.9				
Male	>66	Never	Events (within 1 Year)	1	5	7.54	0.026	0.02	Yes
			Population Time	8.1	303.7				
Male	>66	Past	Events (within 1 Year)	3	36	7.46	0.011	0.001	Yes
			Population Time	10.9	972.1				
Male	>66	Current	Events (within 1 Year)	1	7	5.68	0.025	0.18	No
			Population Time	4.4	174.8				

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ZEUS Scientific

ZEUS Scientific, Inc.
200 Evans Way, Branchburg, New Jersey, 08876, USA
Toll Free (U.S.): 1-800-286-2111, Option 2
International: +1 908-526-3744
Fax: +1 908-526-2058
Website: www.zeusscientific.com

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EC REP
EMERGO EUROPE
Prinsessegracht 20
2514 AP The Hague
The Netherlands

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