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Evaluation of recombinant enzyme calibration to harmonized lipoprotein-associated phospholipase A_2 activity results between instruments

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ABSTRACT

Objectives: Enzymatic activity of lipoprotein-associated phospholipase A_2 (Lp-PLA_2) mediates vascular inflammation in coronary heart disease (CHD). Calibration of Lp-PLA_2 activity measurements using a recombinant enzyme was performed to assess intra- and inter-laboratory assay precision and accuracy in routine clinical settings.

Design and methods: Test performance assessment included recovery, analytical sensitivity, linear range, within-lab and site-to-site precision, interference, and analyte stability. Results using the Beckman-Coulter AU400 analyzer were compared to other chemistry analyzers.

Results: Lp-PLA_2 activity ranged from 84 to 303 nmol/min/mL in 300 subjects, with 82.0% and 18.0% measurements below and at or above a cut-point of 225 nmol/min/mL, respectively. Results of matched K_2-EDTA plasma and serum ($n = 131$) were similar with a slope of 1.00, y-intercept of 0.05, and R-value of 0.988. Mean recovery ranged from 90 to 106% of baseline after storage at different temperatures and time periods. Limit of detection was ≤10 nmol/min/mL, without deviation from linearity between 10 and 382 nmol/min/mL. Endogenous substances and medications did not interfere with the activity measurements. Overall intra- and inter-laboratory precision among three sites showed coefficients of variation of ≤3.8% and ≤5% respectively. Limit of quantitation was 1.3 nmol/min/mL. Method comparison studies for multiple analyzers demonstrated slopes, intercepts or R^2 coefficients ranging from 0.96 to 1.06, −5.6 to 2.0, or 0.997 to 0.999, respectively.

Conclusion: Analytical performance of the calibrated PLAC® test for Lp-PLA_2 enzyme activity assay in CHD resistant to a wide variety of pre-analytical factors, with site-to-site reproducibility on multiple analyzers sufficient to standardize results in diverse laboratory settings.

1. Introduction

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a circulating enzyme whose activity contributes to atherosclerotic cardiovascular diseases (CVD) [1,2] and may be assayed by its cleavage of synthetic substrates containing the *sn-2* position of endogenous phospholipid substrates [3]. Lp-PLA_2 contributes to plaque development by cleaving oxidized LDL to yield lysophatidylcholine and oxidized free fatty acids, which are pro-inflammatory and pro-apoptotic in the atheromatous environment of the arterial wall [3–5]. Clinical studies have demonstrated that Lp-PLA_2 enzyme activity is useful in patient management since levels predict CVD events associated with coronary heart disease (CHD) and ischemic stroke, not only in patients with risk factors alone (primary prevention), but also in those with established disease (secondary prevention) [6]. The Lp-PLA_2 Studies Collaboration analyzed 32 studies and highlighted differences in Lp-PLA_2 enzyme activity levels, which varied primarily due to the use of different substrates [6]. These data suggest that clinical use of Lp-PLA_2 enzymatic activity measurements for patient management may be optimized by assay harmonization.

Inter-laboratory agreement of enzyme activity measurements is a goal in laboratory medicine due to variability in results among different laboratories [7]. Enzymes are a special class of analyte requiring standardization of measurements since catalytic activity depends on testing conditions, and measurements may vary substantially between laboratories due to changes in reaction conditions. Such variability may

Abbreviations: ASA, acetylsalicylic acid; CHD, coronary heart disease; CVD, cardiovascular disease; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantitation; Lp-PLA_2, lipoprotein-associated phospholipase A_2; rLp-PLA_2, recombinant Lp-PLA_2.

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diminish result comparability and hinder clinician interpretation of results [7], particularly in the context of published assay performance data. The magnitude of activity measurements depends on the components of the reaction system and the analytical conditions, but may be optimized by use of a reference measurement procedure. Two tests for Lp-PLA_2 measurements include the same recombinant Lp-PLA_2 (rLp-PLA_2) protein [8–11] for calibrating measurements of enzyme concentration by ELISA [12] or enzyme activity by colorimetric detection of cleaved synthetic substrate. An additional requirement for enzyme activity standardization is a reference interval based on use of a reference measurement system and clinical populations with similar characteristics to obviate potential influences due to ethnicity and/or environment [7].

The clinical value of Lp-PLA_2 activity measurements depends on broad integration of knowledge of the enzyme's physiology, inflammatory role, demographic variation, and clinical practice, together with interpretation of assay results optimized for CVD outcomes. In this work, we report the use of a recombinant Lp-PLA_2 enzyme to calibrate results of the PLAC® Test for Lp-PLA_2 activity, which utilizes an activity cut-point of 225 nmol/min/mL targeted for use in primary prevention of CHD. The objective of this study is to demonstrate the analytical performance of the activity assay, including assay precision, sensitivity, linearity, specimen stability, and site-to-site variability, thus satisfying requirements [7] for use as a standardized enzyme activity assay on a variety of automated chemistry analyzers in diverse clinical laboratory settings.

2. Materials and methods

2.1. Specimens

Human plasma and serum were obtained as archival or fresh samples from vendors (Bioreclamation IVT, LAB Express, and ProMedDx) who collected blood samples from consented donors, or as prospective samples from human volunteers (Diadexus) who provided informed consent, under protocols approved by an Institutional Review Board of participating facilities using standard operating procedures. Demographic information was obtained for each subject and was limited to age, gender, and race; no other clinical information was obtained. Details of specimen handling and storage are provided in the Supplemental information.

2.2. Lp-PLA_2 enzyme activity

Measurement of plasma or serum Lp-PLA_2 activity was performed with the PLAC Test for Lp-PLA_2 Activity (Diadexus) using recombinant Lp-PLA_2 (rLp-PLA_2) enzyme as a standard for calibration as previously described [9]. rLp-PLA_2 was manufactured with proprietary procedures and used to develop a set of master calibrators to standardize each production lot due to the absence of a recognized national reference standard. Patient samples, calibration standards, or high and low controls were added to a reaction buffer containing the substrate, 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine. Substrate hydrolysis by Lp-PLA_2 yielded 4-nitrophenol, which was measured spectrophotometrically using the Beckman Coulter AU400 clinical chemistry analyzer with optimized, instrument-specific settings at 410 nm and an assay time of 8.5 min. Five rLp-PLA_2 calibrators were used to generate a standard curve fit of change in absorbance versus Lp-PLA_2 activity level in nmol/min/mL. This standard curve was utilized to determine sample Lp-PLA_2 activity.

Calibrated sample results were compared to uncalibrated results using two different lots of substrate. A slope from a non-enzymatic curve of absorbances of known solutions of 4-paranitrophenol (PNP) plotted by nmol was used to convert uncalibrated results to Activity units. Sample results were calculated by the following equation: Lp-PLA_2 activity nmol/min/mL = sample kinetic slope/PNP slope/0.025 mL.

2.3. Sensitivity and linearity

The limit of blank (LoB) and limit of detection (LoD) were determined using calibrators and native K_2EDTA plasma, respectively, using analytical approaches described in CLSI EP17-A2 [13], and limit of quantitation (LoQ) was determined using human plasma and non-human plasma dilutions (see Supplemental information).

To determine assay linearity, Lp-PLA_2 activity was measured in triplicate with reagents from three kit lots using a specimen dilution series at 13 different levels for each pair of high and low samples. Three high (~380 nmol/min/mL) and three low (<10 nmol/min/mL) Lp-PLA_2 activity specimens were used from individual donors. To generate high activity specimens for this study, rLp-PLA_2 protein was spiked into specimens with high native Lp-PLA_2 activity. To generate specimens with low activity, patient specimens with low native Lp-PLA_2 activity were diluted with chicken plasma (Bioreclamation IVT, P/N CHLPLEDTA2) to yield Lp-PLA_2 activity values of <10 nmol/min/mL.

2.4. Sample matrix

Freshly drawn blood samples were collected from 21 donors in five different tube types with serum and varying anticoagulant (K2, K3 EDTA and heparin). After collection processing, sample aliquots were stored at 2–8 °C for one day, and then tested in triplicate. For data analysis, the three replicates for each tube type were averaged and then compared to K_2_EDTA plasma results obtained without a separator using a calculated percent recovery and Deming regression. The activity range of the samples, 94 to 323 nmol/min/mL, encompassed 95% of the observed expected values range.

2.5. Assay robustness

To determine assay robustness, Lp-PLA_2 activity measurements were performed on serum samples from 148 subjects using one reagent lot in the absence or presence of five rLP-PLA_2 calibrators ranging from 0 to 400 ng/mL, using two different lots of the substrate, 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine.

2.6. Lp-PLA_2 enzyme activity cut-point

In order to determine clinical performance for the prognostication of future CHD events, an Lp-PLA_2 activity cut-point of 225 nmol/min/mL was established to enable assignment of clinically relevant high or low values. For reference, a value of 225 nmol/min/mL was derived from the JUPITER Study [14] based on determinations of Lp-PLA_2 activity using archival plasma samples previously stored at ≤−70 °C for this study.

2.7. Specimen stability

To determine the effects of temperature and time on the stability of the Lp-PLA_2 activity result, prospectively-collected specimens from 26 subjects were assayed on the day of collection with results used as the "baseline"; then these same samples were analyzed after they were stored at different temperatures for varying time periods. To assess the stability of Lp-PLA_2 activity in unfrozen or frozen specimens, Lp-PLA_2 activity was measured after specimen storage at 4 °C or 26 °C for up to three months or at −20 °C or −70 °C for up to 26 months, respectively. For freeze/thaw study samples, the measured activity value after each freeze thaw cycle was compared to the fresh unfrozen sample value. Measurements of Lp-PLA_2 activity for all temperatures and time intervals were compared to baseline results to determine average recovery values.

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2.8. Interference testing

To determine whether substances may alter the Lp-PLA_2 activity result, measurements were performed to assess both endogenous (albumin, bilirubin (conjugated and unconjugated), cholesterol, hemoglobin, and triglycerides) and exogenous (acetaminophen, acetylsalicylic acid, atorvastatin, clopidogrel bisulfate, diphenhydramine, fenofibrate, lisinopril, metformin, niacin, tolbutamide, warfarin, and vitamin C) substances by spiking each substance into four K_2-EDTA plasma and four serum specimens (see Supplemental Information).

2.9. Reagent stability

Assessments of reagent stability, open bottle stability, and on board analyzer stability were performed to determine their effects on Lp-PLA_2 activity measurements (see Supplemental Information).

2.10. Assay precision

To determine intra-laboratory precision, Lp-PLA_2 activity was measured using four plasma specimens and two kit control samples containing buffer and rLp-PLA_2 activity ranging from 113 to 315 nmol/min/mL. Each run of measurements was performed in duplicate using reagents from three kit lots, with the performance of two runs per day for a 20 day period, yielding 80 results for each kit lot. To determine inter-laboratory (site-to-site) reproducibility, different operators at three independent laboratories assayed Lp-PLA_2 activity in the same set of four K_2EDTA and four serum specimens with Lp-PLA_2 activity ranging from 22 to 380 nmol/min/mL. Assays were performed at each laboratory daily for five days over a two week period using the same kit lot.

2.11. Method comparisons

To assess the performance of the Lp-PLA_2 activity assay on different chemistry analyzers, 40 specimens with Lp-PLA_2 activity values ranging from 5 to 365 nmol/min/mL were tested on various analyzers optimized with instrument specific settings. Data for each analyzer were compared to those yielded by the Beckman Coulter AU400 analyzer by Deming regression analysis [16].

2.12. Statistical analysis

To determine the limit of quantitation (LOQ), precision profiles using CLSI EP17-A2 guidelines [13] and a target coefficient of variation (CV) of 20% were used. To determine assay linearity, Lp-PLA_2 activity results were compared to expected values using linear regression, and the degree of non-linearity was determined for each data set by second and third order polynomial regression plots using CLSI EP06-A guidelines [15]. A t-test was performed to determine the statistical significance of the non-linear coefficients for selection of best polynomial fit based on the lowest standard error of regression. To calculate deviation from linearity for each concentration, the best fit linear y-value was subtracted from the best fit polynomial y-value at that level with conversion of a difference to a percent value. Matrix and method comparison studies were analyzed by Deming regression [16].

To estimate the variance components for each parameter in the precision analyses, the restricted maximum likelihood (REML) method and a random effects ANOVA model were used, with the latter performed separately for each kit lot. To evaluate site-to-site precision, analyses were performed using JMP software (version 9.0.2) and nested linear components of variance model including two factors, "site" and "day," with "day" nested within "site" following CLSI EP05-A3 guidelines [17].

For the cut-point determination, the JUPITER investigators assessed the cardiovascular risk by Cox proportional hazard regression to calculate hazard ratios across quartiles for Lp-PLA_2 Activity by a model minimally adjusted for LDL-cholesterol and a multivariate model adjusted for age, sex, smoking status, family history of premature atherosclerosis, body mass index, systolic blood pressure, HDL-cholesterol and hsCRP.

3. Results

3.1. Analytical performance

Determination of the expected value ranges for Lp-PLA_2 activity for the general population was performed using archival K_2EDTA plasma specimens obtained from 300 random subjects with age ranging from 35 to 75 years (median = 55.7 years). The population evaluated included a gender distribution of 51% men and 49% women and a racial distribution of 38% Caucasian, 32% African-American, 21% Hispanic and 9% Asian subjects. The central 95th percentile distribution of Lp-PLA_2 activity was 84 to 303 nmol/min/mL for all subjects, and 92 to 329 nmol/min/mL or 74 to 300 nmol/min/mL for men or women, respectively, as shown in Table 1.

Analysis of plasma specimens from 5446 subjects associated with primary prevention of CHD in the placebo arm of the JUPITER Study [14] demonstrated that Lp-PLA_2 activity in the highest quartile was associated with a hazard ratio (HR) for CHD events of 2.83 (C.I. = 1.63–4.94) or 2.15 (C.I. 1.13–4.08) in minimally-adjusted or fully-adjusted models, respectively. These data enabled the derivation of a cut-point of 225 nmol/min/mL to differentiate those at either increased, i.e., high Lp-PLA_2 (≥225 nmol/min/mL), or decreased CHD event risk, i.e., low Lp-PLA_2 (<225 nmol/min/mL) risk for CHD events. Use of the cut-point identified 82.0% or 18.0% of the 300 subjects with low or high activity, respectively; similarly, the cut-point identified 79% and 85% of men and women, respectively, with low activity, and 21% and 15% of men and women, respectively, with high activity. No clinical information, i.e., health or disease status, was available for the analysis.

Further analyses yielded an LOB of 0.4 nmol/min/mL, an LOD of 0.74 nmol/min/mL, and an LOQ of 1.3 nmol/min/mL. Use of weighted linear regression to analyze assay linearity yielded a range of 6 to 382 nmol/min/mL, as shown in Table S1, with deviation from linearity less than or equal to 10%. Assessment of intra-laboratory variability using three kit lots during a 20 day period in the Lp-PLA_2 activity range of 113 to 315 nmol/min/mL yielded the following within-laboratory composite CVs: within-run, ≤1.5%; between-run, ≤1.8%; day-to-day, ≤1.5%;

Table 1
Expected Lp-PLA_2 activity values.

Percentile	Lp-PLA_2 activity (nmol/min/mL)		
	All	Male	Female
	(n = 300)	(n = 154)	(n = 146)
Minimum	50	70	50
2.5	84	92	74
5	94	102	86
20	129	144	123
25	137	149	130
33	148	155	139
50	167	176	154
67	196	204	179
75	211	219	200
80	221	232	215
95	276	295	264
97.5	303	329	300
99	369	397	339
Maximum	>400	>400	370
Mean	176	186	166
S.D.	58	59	55

Shown are the expected minimum, maximum and mean values and also the values at the indicated percentiles for Lp-PLA_2 enzyme activity (nmol/min/mL) for the entire subject cohort and by gender. (S.D., standard deviation).

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lot-to-lot, ≤3.1%; and total precision (all variables), ≤3.8%. Lp-PLA_2 activity was measured using two different substrate lots in the absence of rLp-PLA_2 calibration. Results were converted to activity units with a factor derived from a non-enzymatic measurement using 4-nitrophenol, as previously described [6,20,21]. Results showed an 18% difference in measurements; no statistically significant difference was observed between the lots using rLp-PLA_2 calibration, as shown in Fig. 1.

3.2. Sample matrix

To assess potential variability associated with blood specimen types or plasma collection protocols, Lp-PLA_2 activity was measured using matched serum and plasma specimens, as well as matched plasma specimens in different blood collection tubes. As shown in Fig. 2, matched serum and K_2EDTA plasma data from 131 subjects demonstrated a slope of 1.00 (95% C.I.: 0.974 to −1.029) in the range of 56 to 357 nmol/min/mL, with an intercept of 0.05 (95% C.I.: -1.28 to 1.39) and an R coefficient of 0.988 by Deming regression analysis [16]. These data demonstrated comparable Lp-PLA_2 activity results for matched serum or plasma specimens.

To assess the effect of using different blood collection tubes, Lp-PLA_2 activity was measured in specimens obtained from 21 subjects distributed among tubes containing K_2EDTA, K_2EDTA gel, K_3EDTA, and lithium heparin for plasma, and also serum tubes with and without separator gel. Comparison of the matched K_2EDTA gel, K_3EDTA, and lithium heparin results with those for K_2EDTA or serum showed slopes ranging from 1.00 to 1.03 with intercepts ranging from −1.03 to 0.93 and R coefficients ranging from 0.997 to 0.999, thus demonstrating the independence of the Lp-PLA_2 activity result from blood collection tube type, as shown in Table S2.

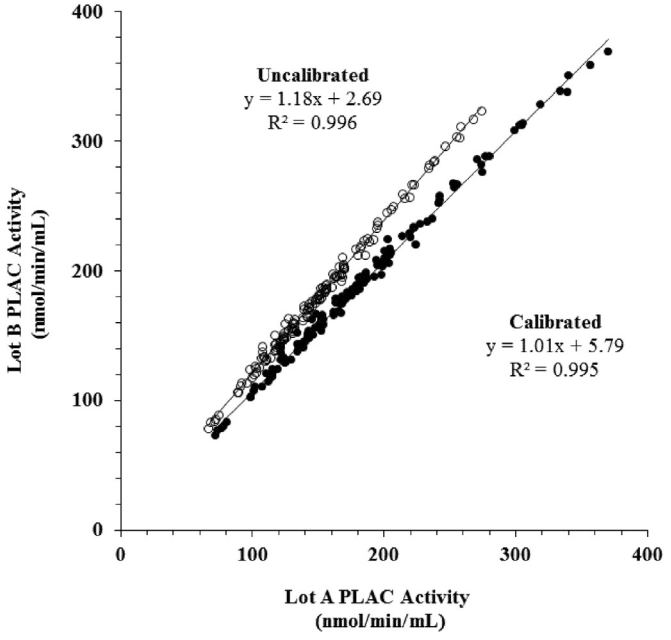

Fig. 1. Comparison of Lp-PLA_2 activity measurements without and with rLp-PLA_2 calibration. Lp-PLA_2 activity was determined for 139 serum specimens using two different substrate lots, A and B, without (uncalibrated, open circles) or with (calibrated, filled circles) use of rLp-PLA_2 calibrators. Activity units for both data sets are expressed in units of nmol/min/mL. The calibrated results were based on quantitation from a standard curve using Lp-PLA_2 enzyme calibrators and the uncalibrated results were calculated utilizing a factor based on the slope of 4-nitrophenol absorbance at various concentrations. Regression parameters from the results using lots A and B for either uncalibrated or calibrated data, and the relative recovery rate (%) between the two lots are shown.

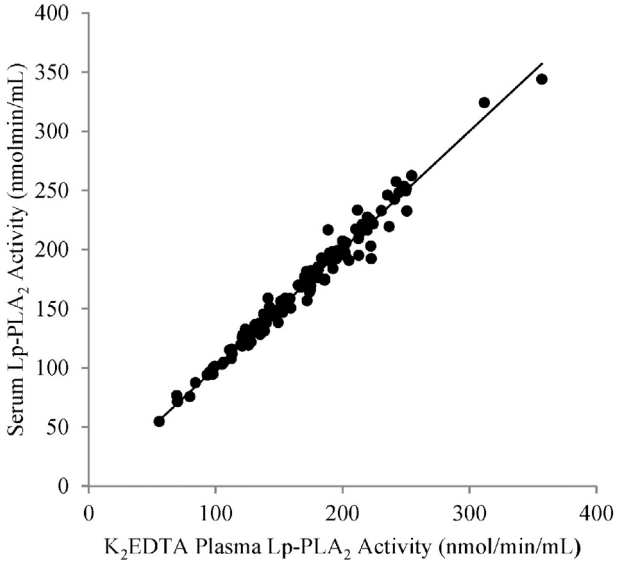

Fig. 2. Comparison of K_2EDTA and serum Lp-PLA_2 activity measurements. Lp-PLA_2 activity measurements (nmol/min/mL) were determined for 131 matched K_2EDTA plasma and serum samples from individual donors and plotted as shown. The data demonstrated a slope of 1.00, an intercept of 0.05 nmol/min/mL, and an R value of 0.988 by Deming regression [16].

3.3. Sample storage

Since some enzymatic proteins demonstrate lability during storage, the potential variability in Lp-PLA_2 activity results due to specimen storage conditions over time was evaluated. Assessment of K_2EDTA plasma specimens at 4 °C for up to three months and at 26 °C for up to one month demonstrated average recovery values of greater than or equal to 95% for Lp-PLA_2 activity compared to those at baseline, as seen in Table 2. Lp-PLA_2 activity ranged from 62 to 239 nmol/min/mL. Evaluation of frozen storage conditions for K_2EDTA plasma obtained from 10 subjects and stored at −20 °C or −70 °C for durations of up to 26 months showed differences of less than 10% upon comparison of the average recovery at each time point compared to the baseline value, as shown in Table 2. No statistically significant differences were observed for values between serum and K_2EDTA plasma after frozen storage (data not shown). Similarly, serum and K_2EDTA plasma specimens (n = 20) subjected to seven freeze–thaw cycles demonstrated average recovery of 95 to 100% at −20 °C and 96 to 97% at ≤− 70 °C compared to baseline Lp-PLA_2 activity values.

3.4. Substance interference

Since patients with CHD commonly have associated conditions and use medications as part of routine management, the potential variability

Table 2
Lp-PLA_2 activity stability by storage conditions.

Time	2–8 °C (n = 26)	26 °C (n = 26)	−20 °C (n = 10)	−70 °C (n = 10)
Day 1	100%	103%	102%	100%
Day 7	95%	102%	97%	98%
Month 1	95%	102%	106%	106%
Month 2	96%	78%	91%	90%
Month 3	97%	70%	101%	102%
Month 13	NA	NA	98%	94%
Month 26	NA	NA	95%	100%

Average recovery values are shown for Lp-PLA_2 activity measurements for the indicated temperatures and time intervals expressed as a percentage of baseline values. (NA, not applicable).

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of Lp-PLA$_2$ activity due to both endogenous and exogenous substances was evaluated. As shown in Table S3, none of the blood components or medications assessed, including those related to CHD, demonstrated interference as shown by analyses of average recovery for spiked and corresponding control samples.

3.5. Inter-laboratory performance

To assess the inter-laboratory variability associated with the Lp-PLA$_2$ activity assay, site-to-site reproducibility for three different laboratories was evaluated. Site-to-site reproducibility in the Lp-PLA$_2$ activity range of 22 to 380 nmol/min/mL during a 5 day period yielded individual within-lab precision CVs of 1.4 to 4.2%, as shown in Table 3. Analysis of site-to-site precision demonstrated CVs ≤ 3.4% for all samples and total precision CVs ranging from 2.7 to 4.6%.

Since the Lp-PLA$_2$ activity assay kit may be used in a variety of laboratory settings, its reproducibility was assessed by testing 40 samples on different automated chemistry analyzers and comparing the results to those observed for the Beckman Coulter AU400 analyzer. As seen in Table 4, method comparison analyses of the analytical performance of the Lp-PLA$_2$ activity assay using 11 additional chemistry analyzers demonstrated slopes between 0.96 and 1.06 with correlation coefficients (R^2) greater than or equal to 0.997. Analysis of once opened and stored, capped reagents at 4 °C from three kit lots at 16 weeks demonstrated average percent recovery ranging from 90 to 110% compared to baseline values. Similarly, use of reagents that were stored uncapped on-board automated clinical chemistry analyzers for 6 weeks demonstrated average recovery ranging from 90 to 110% compared to baseline values.

4. Discussion

Clinical interpretation of Lp-PLA$_2$ enzyme activity measurements in the management of patients at risk for CHD may be optimized by assay standardization to enhance inter-laboratory agreement and correlation of individual patient results to those observed in published clinical performance data. The clinical use of enzyme activity measurements requires optimization by assay standardization due to myriad contributors to results variability [7], including the following: individual or population heterogeneity phenotypes; specimen collection, handling and storage protocols; assay interference by endogenous or exogenous substances; use of different analytical platforms; differences in raw materials; and differences in laboratory protocols. To enable standardization of measurements of Lp-PLA$_2$ enzyme activity for patient management, the PLAC Test for Lp-PLA$_2$ Activity kit includes ready-to-use reagents and a recombinant Lp-PLA$_2$ (rLp-PLA$_2$) enzyme for use as calibrators and controls. In the present study, we demonstrate that using rLp-PLA$_2$ calibrators traceable and consistent to reference material quantitate reproducible Lp-PLA$_2$ activity assay results, enabling use of patient specimens from different medical practice environments and performance on different automated chemistry analyzers in diverse clinical laboratory settings.

The Lp-PLA$_2$ enzyme activity assay demonstrated analytical performance sufficient for correlating patient results to data in published clinical studies of Lp-PLA$_2$ activity in CVD [14]. The analyses yielded an LOQ of ≤10 nmol/min/mL for analytical sensitivity, which is sufficient to support clinical measurements observed for the CHD population, and an assay linearity range of 6 to 382 nmol/min/mL, which is similar to that reported in an independent analysis [18]. The central 95th percentile of expected Lp-PLA$_2$ activity values ranged from 84 to 303 nmol/min/mL, with a higher median value of 176 nmol/min/mL observed for men compared to the 154 nmol/min/mL observed for women. Use of an enzyme activity cut-point of 225 nmol/min/mL to determine low (below cut-point) or high (at or above cut-point) activity levels yielded a distribution of 82% and 18%, respectively, for the population studied, while distributions of low and high levels for men or women were 79% or 85% and 21% or 15%, respectively. Comparisons of Lp-PLA$_2$ activity measurements using two substrate lots, without or with rLp-PLA$_2$ calibrators, demonstrated that rLp-PLA$_2$ calibration increased assay reproducibility (Fig. 1). Interference testing demonstrated that neither endogenous circulating substances nor medications typically used by patients at risk for CHD altered Lp-PLA$_2$ activity results, as shown in Table S3. While differences in blood matrices may influence activity results of enzymes, neither use of serum or plasma, nor blood collection in different tube types yielded statistically significant differences in Lp-PLA$_2$ activity results. Variations in sample handling with respect to storage in unfrozen or frozen conditions for clinically relevant time periods did not yield statistically significant differences in Lp-PLA$_2$ activity measurements. Moreover, Lp-PLA$_2$ enzyme activity measurements remained stable after multiple freeze–thaw cycles. Therefore, potential variations due to pre-analytical specimen handling do not affect calibrated Lp-PLA$_2$ enzyme activity measurements, in contrast to a prior report for determinations of Lp-PLA$_2$ concentration by ELISA [19].

Analyses of assay performance on different automated chemistry analyzers with ready-to-use reagents in different laboratories also demonstrated that calibrated Lp-PLA$_2$ enzyme activity measurements achieved requirements necessary for inter-laboratory agreement and standardized interpretation of activity results stipulated by the International Federation of Clinical Chemistry and Laboratory Medicine [7]. Comparison studies of Lp-PLA$_2$ activity results performed on multiple automated chemistry analyzers demonstrated Deming regression line slopes of 0.96–1.06 and correlation coefficients greater than or equal to 0.997, thus supporting the independence of the measurements from variation in accuracy due to analysis on various analyzers commonly used in different laboratories. The demonstration of CVs less than or equal to 3.4% for all samples tested on the same analyzer in three different laboratories by different operators also showed the site-to-site reproducibility of calibrated Lp-PLA$_2$ activity results. The contribution of ready-to-use

Table 3
Analysis of intra-and inter-laboratory precision for K_2EDTA plasma and serum specimens.

Sample	Mean	Within-lab	Site-to-site	Total
	(nmol/min/mL)	CV	CV	CV
Plasma 1	21.9	2.6%	2.7%	3.8%
Plasma 2	108.3	2.1%	2.8%	3.5%
Plasma 3	233.6	1.8%	1.9%	2.7%
Plasma 4	379.5	1.4%	2.3%	2.7%
Serum 1	23.6	4.2%	1.9%	4.6%
Serum 2	102.3	1.7%	3.4%	3.8%
Serum 3	232.3	2.1%	3.1%	3.7%
Serum 4	355.9	2.3%	2.2%	3.2%

Shown are the mean Lp-PLA$_2$ activity results, the within-lab coefficient of variation (CV), the site-to-site CVs using the Beckman Coulter AU400 analyzer at three independent sites, and the total CV for each of four K_2EDTA plasma and four serum specimens.

Table 4
Method comparison of automated clinical chemistry analyzers.

Clinical chemistry analyzer	Slope	Intercept (nmol/min/mL)	R^2 value
Abbott ARCHITECT c8000/ci8200	1.06	−3.47	0.999
Beckman Coulter AU480	1.00	−3.35	0.999
Beckman Coulter AU640	0.96	2.03	0.999
Beckman Coulter AU5800	0.99	−0.90	0.999
Beckman Coulter DxC600	1.01	−2.38	0.999
Beckman Coulter DxC800	1.03	−4.73	0.999
Roche cobas 6000 (c501)	1.03	−4.48	0.999
Roche cobas 8000 (c502)	1.05	−3.31	0.997
Roche Modular-P	1.05	−5.62	0.999
Siemens Advia 1800	0.99	1.74	0.998
Siemens Advia 2400	0.96	0.58	0.999

Shown are the slopes, intercepts (nmol/min/mL), and R^2 coefficient values for Lp-PLA$_2$ activity results obtained using the eleven indicated clinical chemistry analyzers with data for each compared to those obtained using the Beckman Coulter AU400 analyzer.

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kit reagents to site-to-site precision was also shown by the average recovery ranging from 90 to 110% of established values, demonstrating the open bottle stability of the reagents for up to 6 weeks for use in different laboratory operating protocols. The open bottle study demonstrated stability of uncapped on board buffers and substrate reagents for up to 6 weeks.

In summary, the PLAC Test for Lp-PLA_2 Activity kit utilizes calibration by a recombinant Lp-PLA_2 protein with ready-to-use reagents to achieve high intra-laboratory and inter-laboratory precision, and also the requirements stipulated for enzyme activity assay standardization [7]. The data also demonstrate that a calibrated Lp-PLA_2 activity measurement is independent of heterogeneity in the therapeutic management of patients at risk for CVD and in pre-analytical processes such as blood collection and handling common to diverse practice settings. The clinical value of a standardized Lp-PLA_2 enzyme activity assay is that it may 1) enhance physician interpretation of activity measurements by enabling correlation of an individual patient's result to extensive, published clinical validation data for CVD population outcomes, and 2) enables harmonization of results obtained on patients from different laboratories with varying operating protocols.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2015.11.018.

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