

Development and validation of an isoform-independent monoclonal antibody–based ELISA for measurement of lipoprotein(a)

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The study aims were to develop a new isoform-independent enzyme-linked immunoassay (ELISA) for the measurement of lipoprotein(a) [Lp(a)], validate its performance characteristics, and demonstrate its accuracy by comparison with the gold-standard ELISA method and an LC-MS/MS candidate reference method, both developed at the University of Washington. The principle of the new assay is the capture of Lp(a) with monoclonal antibody LPA4 primarily directed to an epitope in apolipoprotein(a) KIV₂ and its detection with monoclonal antibody LPA-KIV9 directed to a single antigenic site present on KIV₉. Validation studies were performed following the guidelines of the Clinical Laboratory Improvement Amendments and the College of American Pathologists. The analytical measuring range of the LPA4/LPA-KIV9 ELISA is 0.27–1,402 nmol/L, and the method meets stringent criteria for precision, linearity, spike and recovery, dilutability, comparison of plasma versus serum, and accuracy. Method comparison with both the gold-standard ELISA and the LC-MS/MS method performed in 64 samples with known apolipoprotein(a) isoforms resulted in excellent correlation with both methods ($r=0.987$ and $r=0.976$, respectively). Additionally, the variation in apolipoprotein(a) size accounted for only 0.2% and 2.2% of the bias variation, respectively, indicating that the LPA4/LPA-KIV9 ELISA is not affected by apolipoprotein(a) size polymorphism. Peptide mapping and competition experiments demonstrated that the measuring monoclonal antibodies used in the gold-standard ELISA (a-40) and in the newly developed ELISA (LPA-KIV9) are directed to the same epitope, ⁴⁰⁷⁶LETPTVV⁴⁰⁸², on KIV₉. In conclusion, no statistically or clinically significant bias was observed between Lp(a) measurements obtained by the LPA4/LPA-KIV9 ELISA and those obtained by the gold-standard ELISA or LC-MS/MS, and therefore, the methods are considered equivalent.

Supplementary key words lipoprotein(a) • monoclonal antibody • isoform • kringle • cardiovascular disease • aortic stenosis • metabolism • therapy • LPA-KIV9

Lipoprotein(a) [Lp(a)] is an apo B-containing lipoprotein that is a genetic, independent risk factor for cardiovascular disease and aortic stenosis (1). Lp(a) is similar in composition to LDL but additionally characterized by the presence of a carbohydrate-rich protein termed apolipoprotein(a) [apo(a)] that is covalently linked by a disulfide bond to the single molecule of apoB-100. The presence of apo(a) imparts specific pathophysiological and metabolic characteristics to Lp(a) rendering it significantly different from LDL (2).

Apo(a) is formed by repeated kringle (K) structures (KIV), a single copy of KV, and an inactive protease domain, all possessing a high amino acid sequence homology with the corresponding structure of plasminogen. In apo(a), KIV is formed by 10 different subtypes (KIV₁ to KIV₁₀), each present as a single copy with the exception of KIV₂ which is present in a highly variable number in different individuals ranging from 1 to >40 copies of identical repeats. The repeats are due to copy number variations in the *LPA* gene, and therefore, individuals may inherit highly different apo(a) molecular weight ranging from ~300 to 800 kDa. The variation in the number of KIV₂ gives origin to the >40 apo(a) isoforms circulating in human plasma of different individuals and is primarily responsible for the size heterogeneity of Lp(a). The concentration of Lp(a) is also highly heterogeneous, varying >1000 fold within the population, and to a major extent is genetically controlled and inversely related to the copy number variation in the *LPA* gene (2).

The large size heterogeneity of apo(a) has been a major challenge to the immunochemical measurement of Lp(a) because the variable number of repeated KIV₂ motifs results in a variable number of antigenic

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epitopes in the samples to be analyzed. Consequently, plasma levels of Lp(a) will be overestimated or underestimated in test samples when the number of KIV₂ is higher or smaller than those present in the assay calibrator (3).

Ideally, use of monoclonal antibodies directed to a single antigenic site on apo(a) will be able to solve the impact of the variable number of KIV₂. However, the high homology of the apo(a) kringles (~75–94%) (4) has proven to be highly challenging in developing monoclonal antibodies binding to a unique epitope not present in KIV₂.

In 1995, Marcovina *et al.* described the production of a monoclonal antibody (a-40) directed to a unique epitope located in KIV₉ of apo(a) and its use as a detecting antibody in the development of an enzyme-linked immunoassay (ELISA) demonstrated to accurately measure Lp(a) without the impact of the apo(a) size polymorphism in the samples (5). Because this ELISA does not measure the variable mass of Lp(a) but the number of circulating particles, the Lp(a) concentration is expressed in nmol/L. However, while this ELISA has been extensively used in research and for assay standardization as a “gold standard”, the assay has never been made available outside of the University of Washington.

A monoclonal antibody (LPA-KIV9), also directed to KIV₉, was recently generated at the University of California San Diego and extensively evaluated as previously reported (6). The aim of the current study was to develop a new sandwich Lp(a) ELISA modeled on the approach used by Marcovina *et al.* (5) and based on two monoclonal antibodies LPA-KIV9 (6) and LPA4 (7). To demonstrate its performance characteristics, we report here the extensive validation of this assay.

MATERIALS AND METHODS

Antibodies

The generation and characterization of murine IgG monoclonal antibodies LPA4 and LPA-KIV9 were previously described (7). Briefly, LPA4 was generated by immunizing mice with the 14-amino acid peptide TRNYCRNPDAEIRP present on apo(a) KIV₅, KIV₇, and KIV₈. However, the partial sequence NYCRNPDA is also present on KIV₂ and appears to be immunologically dominant as LPA4 strongly interacts with KIV₂ (8). As previously described, monoclonal antibody LPA-KIV9 was generated by immunizing mice with a truncated recombinant apo(a) formed by eight kringle KIV repeats containing one copy of KIV₁, one copy of KIV₂, a fusion of KIV₃ and KIV₅, individual KIV₆ to KIV₁₀, KV, and the protease domain (6, 9). As reported, LPA-KIV9 has been shown to bind to sequence ⁴⁰⁷⁶LETPTVV⁴⁰⁸² on KIV₉, which is present only once on apo(a) (6).

Double-antibody sandwich ELISA

The development of the ELISA method and the validation studies were performed at Medpace Reference

Laboratories Cincinnati facility. This sandwich ELISA is based on the use of LPA4 as the capture antibody and the use of LPA-KIV9 as the detection antibody (Fig. 1). Based on the LETPTVV epitope specificity of LPA-KIV9, this assay is not expected to be affected by the size variation of apo(a) in Lp(a) samples.

Initial attempts to directly bind LPA4 to 96-well plates (MaxiSorp, Thermo Fisher) were not successful, and therefore, LPA4 was biotinylated, and streptavidin coated plates were used. Ninety-six-well microplates were precoated with streptavidin (Pierce® Streptavidin High Binding Capacity Coated 96-Well Plates, Thermo Fisher), and biotinylated LPA4 (Biotinylation Kit / Biotin Conjugation Kit (Fast, Type B)—Lightning-Link®, Abcam) (100 µl at 2 µg/ml) was added to the plates in a saturating amount and incubated for 2 h in a shaker at 300 rpm at room temperature. The WHO/IFCC 1st reference material for Lp(a) immunoassays, SRM-2B, with an assigned value of 107 nmol/L was used as the assay calibrator (10). After reconstitution of the lyophilized reference material, a stock solution was prepared by diluting the material with dilution buffer to a concentration of 4.38 nmol/L. The 8-point standard curve was then prepared by diluting the stock solution x10, x12, x16, x20, x30, x40, x80, and x160 to yield solutions of 0.438, 0.365, 0.274, 0.219, 0.146, 0.110, 0.055, and 0.027 nmol/L, respectively.

After washing and blocking of nonspecific sites with 3% BSA, the diluted standards and test samples diluted at 1:400 were added and incubated in the dark for 1 h at 28°C with shaking at 1000 rpm. To increase the precision of the measurements, selected samples, based on their relative Lp(a) concentrations, were re-analyzed at optimal dilutions (1:10 to 1:3200) so that all the absorbances are confined in the middle and most stable part of the standard curve. The Lp(a) particles present in the standards and in the test samples are bound to the immobilized LPA4 and then detected with HRP-LPA-KIV9. The addition of a chromogenic substrate (OPD Substrate Tablets [o-phenylenediamine dihydrochloride] with 7 µl hydrogen peroxide, Sigma) allows for quantitative determination of HRP activity that is proportional to the amount of captured Lp(a). The plates were read on a Versa max microplate reader (Molecular Devices, San Jose, CA) at a wavelength of 490 nm with background subtraction at 630 nm. The Lp(a) concentrations are reported in nmol/L.

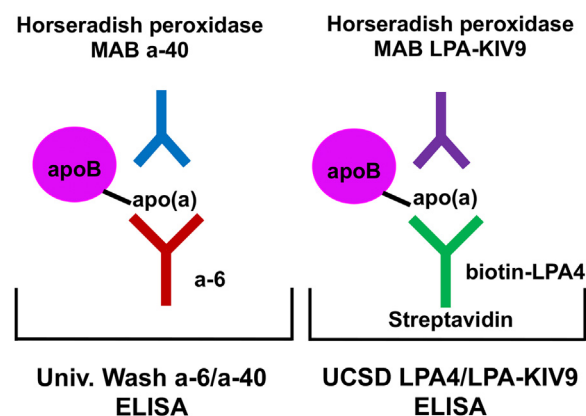


Figure 1. Methodology of the University of Washington and the UCSD ELISAs. The principle of both these sandwich ELISAs is the capture of Lp(a) with monoclonal antibodies that bind to KIV₂ and detection antibodies that bind only once to KIV₉. Lp(a), lipoprotein(a).

Assay validation

The Lp(a) ELISA validation follows the guidelines of the Clinical Laboratory Improvement Amendments and the College of American Pathologists. Method performance characteristics assessed during validation were following the subsections of Clinical Laboratory Improvement Amendments regulation 42CFR493.1253. Acceptability of results throughout the validation were based on the recommendations of the FDA 2018 Bioanalytical Method validation Guidance for ligand binding assays as well as on the concept of total allowable error established using the Westgard Desirable Biological Variation Database (11) specifications for imprecision (10.4%) and bias (6.9%).

Repeatability (within-run imprecision)

Three levels of in-house prepared quality control (QC) pools were analyzed in 22 replicates in a single plate. QC low, QC medium, and QC high were diluted before analysis x50, x400, and x1600, respectively. The replicates were obtained from 11 different dilutions. The repeatability was acceptable if the coefficient of variation (CV) for each level QC was <10%.

Reproducibility (total imprecision)

The same QC samples used in the repeatability testing were analyzed in duplicate at the beginning and end of every plate for 8 days. The assay reproducibility was deemed acceptable if the calculated CV was <15.0%.

Analytical measuring range

The analytical measuring range (AMR), or reportable range of the assay, is the range of analyte concentrations with which the assay can provide measured quantity values that are directly proportional to the value of the measurand in the samples. The AMR was assessed by determining the accuracy and precision throughout the range of the standard curve from 0.027 nmol/L to 0.438 nmol/L. Results were evaluated to determine the lower limit of quantification (LLOQ), limit of detection, and upper limit of quantification (ULOQ) of the assay.

Linearity

Independent dilutions of the assay calibrator were prepared, and each of the eight solutions was analyzed in duplicate as an unknown against the calibration curve for five different times over four different days. Response values were back calculated, and interpolated concentrations were determined. The mean SD, %CV, bias, and %bias from the nominal concentration were calculated from the results of each standard solution.

Limit of quantitation

Testing for the LLOQ and ULOQ was incorporated into the linearity step by evaluating the performance of the assay at the limits of the analytical measuring range.

Spiking and recovery

Spike and recovery experiments were performed to identify the systemic error that may arise from the interaction between other matrix components and Lp(a).

The concentration of Lp(a) in five serum samples from individual donors was determined before (baseline) and after

spiking with two different levels of the WHO/IFCC Reference Material with the assigned value of 107 nmol/L (10). The ratio of spike volume to total sample volume was 1:10 (1 part Lp(a) control material to 9 parts serum) and 1:20 (1 part Lp(a) control material to 19 parts serum). Samples were spiked after the appropriate dilution of all specimens. Each sample was run in duplicate, and the mean, SD, %CV, bias, and %bias were calculated. The recovery was deemed acceptable if the obtained mean %bias was within $\pm 20.0\%$ of the baseline concentration (*i.e.*, recovery of 80.0%–120.0%).

Dilutability

Dilutability experiments were performed to confirm compatibility between the sample diluent and the matrix. The dilutability was performed using five serum samples with known concentration of Lp(a) ranging from 3.2 nmol/L to 343 nmol/L. Multiple dilutions of each sample were performed for their values to fall in the range of the standard curve. Each sample dilution was analyzed four times, results corrected for the dilution, and the mean, SD, %CV, bias, and % bias were calculated. Results were acceptable if the recovery of each measured concentration of the diluted samples, after correcting for the dilution, was between 80% and 120%.

Matrix correlation (serum vs. plasma)

Serum and K2EDTA-plasma samples were collected from 38 volunteer individuals and analyzed in parallel. The two matrixes were considered equivalent if no statistically significant bias was observed. If observed, the bias was considered not clinically significant if it was <15%. Lithium-heparin plasma was not investigated.

Accuracy

In the absence of a reference method for Lp(a), and following the recommendations of the Joint Committee for Traceability and the International Organization for Standardization (11–13), the accuracy of the method was evaluated by comparison of results obtained by the LPA4/LPA-KIV9 ELISA with those obtained by the monoclonal antibody-based ELISA developed by Marcovina *et al.* at the University of Washington, using capture monoclonal antibody a-6 and detection antibody a-40 (5). This method, demonstrated to measure apo(a) and apoB-100 in Lp(a) on an equimolar basis and therefore unaffected by the apo(a) size polymorphism, is considered the “gold standard” method for Lp(a) measurement (3). Additionally, the results of the LPA4/LPA-KIV9 ELISA were compared to those obtained by a targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) candidate reference method for Lp(a) (14). This LC-MS/MS method was rendered independent from the size polymorphism of Lp(a) by selecting three specific quantification peptides not present in the KIV₂ region of apo(a). Accuracy of the measurements were achieved by using a high-purity 14 KIV recombinant apo(a) as a primary reference material to calibrate the assay in SI units.

The evaluation was performed on a set of 64 fresh-frozen samples from individual donors that were previously tested at the University of Washington by the Lp(a) ELISA and by the LC-MS/MS method. Analyses of the 64 samples, spanning a good range of Lp(a) levels and apo(a) isoform size, were performed by the LPA4/LPA-KIV9 ELISA in duplicate over three different days. The methods were considered equivalent if no statistically significant bias was observed. If

observed, the bias was considered not clinically significant if it was <15.0%. Determination of apo(a) isoforms in the 64 comparison samples was performed by a high-sensitive agarose gel electrophoresis method followed by immunoblotting as previously described (13) with each isoform designated by the relative number of KIV.

Determination of the peptide epitope of monoclonal antibody a-40

A peptide library array spanning KIV₉, consisting of 100 overlapping, 15 amino acid peptides differing by only one amino acid residue was synthesized on cellulose membranes (JPT, Germany). The membrane was incubated with monoclonal antibody a-40 (1 µg/ml, overnight at 4°C) and antibody binding detected with an anti-mouse IgG antibody conjugated with horseradish peroxidase and SuperSignal West Dura HRP chemiluminescent substrate (Thermo Scientific), as previously described (6).

To further evaluate whether monoclonal antibodies a-40 and LPA-KIV9 react with the same or different epitopes on KIV₉, a competition experiment was performed with a modified version of the LPA4/LPA-KIV9 assay. In brief, LPA4 was plated overnight in microtiter well plates at 5 µg/ml. A plasma sample with a previously determined Lp(a) value of 170 nmol/L was used and added to the well at 1:400 dilution. Biotin-labeled LPA-KIV9 was then added at 1 µg/ml to bind Lp(a). Plates were washed three times, incubated with alkaline phosphatase-conjugated NeutrAvidin (Thermo Scientific, 1:40,000 dilution) for 60 min at room temperature, washed, and incubated with Lumi-Phos-530 (Lumigen Inc, Southfield, Michigan 1:1 dilution in water) for 75 min at room temperature (25 ml/well) and luminescence measured (Bio-Tek Instruments, Inc, Winooski, VT). Results were displayed as relative light units per 100 milliseconds. The competition assay was performed by co-incubating the diluted plasma sample with 1, 5, and 10 µg/ml of a-40. These experiments were performed at UCSD.

Statistics

SoftMax Pro 7.1 GxP software was used to analyze microplate data generated from the Versa max microplate reader. Microsoft® Office Excel and a statistical software package Analyse-It® (version 3.90.7) were used for validation and statistical calculations. The mean values of the assay

comparison were analyzed using Deming Regression and Bland Altman (15) plots to determine bias. Linearity was determined to evaluate the statistical significance of second- and third-order polynomials in the fit between the expected, theoretical concentration and the observed concentration. If second- or third-order polynomials significantly improved the fit at $P = 0.05$, the deviation from linearity was unacceptable if it was greater than 15.0% (25.0% at LLOQ and ULOQ).

RESULTS

Precision

The intraassay precision for the three level QC samples was excellent with the CVs ranging from 4.2% to 5.8% (supplemental Table S1). The total assay imprecision for the same three level QC samples analyzed in duplicate at the beginning and end of every plate for 8 days was also excellent with total CVs ranging from 6.5% to 9.1% (supplemental Table S2).

Analytical measuring range

Linearity. Eight levels of calibrator solutions ranging from 0.027 nmol/L to 0.438 nmol/L were used to evaluate the linearity of the assay. The precision (CV) ranged from 3.7% to 23.9%, and the bias ranged from -5.9% to 2.1% (supplemental Table S3 and Fig. 2). Second- and third-order polynomials did not improve the fit between the expected and the observed concentrations. The results demonstrate good accuracy and precision over a linear range from 0.027 nmol/L to 0.438 nmol/L, and therefore, the assay AMR is 0.027 nmol/L to 0.438 nmol/L. Accounting for the minimum required dilution of x10, the AMR is 0.27 nmol/L to 4.38 nmol/L. Based on the range of acceptable dilutions up to x3200, as demonstrated in the dilutability experiments, the extended AMR is from 0.27 nmol/L to 1401 nmol/L.

Limit of quantitation. Testing for the LLOQ and ULOQ was incorporated into the linearity step

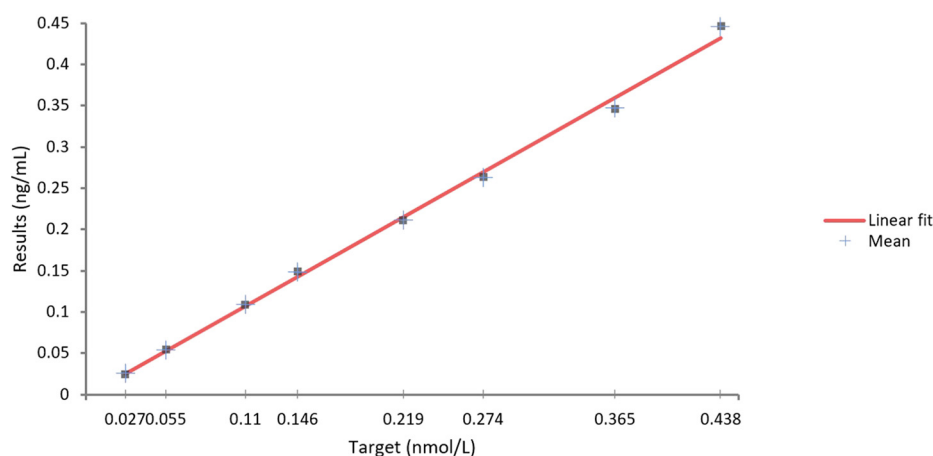


Figure 2. Linearity of eight levels of calibrator solutions ranging from 0.027 nmol/L to 0.438 nmol/L used to evaluate the LPA4/LPA-KIV9 assay.

(supplemental Table S3). The lowest standard that demonstrates acceptable performance as the LLOQ is 0.027 nmol/L (Level 8), with an observed bias and CV of -5.9% and 23.9%, respectively. The bias and CV observed for the highest standard, 0.438 nmol/L (Level 1), were 1.8% and 7.7%, respectively. Using the minimum required dilution of x10, the LLOQ is 0.27 nmol/L, and ULOQ is 4.38 nmol/L.

Spiking and recovery

The bias of Lp(a) after spiking the five samples with two different levels of the reference material ranged from -5.7% to +6.3% (supplemental Table S4). The assay demonstrated an excellent Lp(a) recovery ranging from 94% to 106% with %bias values for all five samples, well below the $\pm 20\%$ acceptance criteria.

Dilutability

A close approximation of Lp(a) levels and their dilutions was observed for the five samples as the % recovery ranged from 85.9% to 118.0% (supplemental Table S5). These results confirm that dilution of the samples to bring the absorbances in the linear range of the standard curve did not cause significantly different results. This allowed the expansion of the range of the standard curve (extended analytical range) as sample dilutions as high as x3200 provided consistently reliable results.

Matrix correlation (serum/plasma)

Deming Regression and Bland-Altman analyses identified a mean bias of 2.02% between the 38 serum and plasma samples with a correlation coefficient of 0.998 (supplemental Fig. S1). The 95% confidence interval around the estimate of the %bias was from -1.22% to +5.26%, which includes 0%, and therefore, it is not statistically significant. Lp(a) measurements can therefore be performed either in serum or in EDTA-plasma.

Accuracy

Comparison of the LPA4/LPA-KIV9 ELISA with the a-6/a-40 "gold standard" ELISA. Deming Regression and Bland-Altman analyses identified a mean bias of 2.73% between the LPA4/LPA-KIV9 ELISA and the a-6/a-40 ELISA with a correlation coefficient of 0.987 (Fig. 3A). The 95% confidence interval around the estimate of the %bias ranged from -0.125% to +5.578%, which includes 0% and is therefore not statistically significant (Fig. 3B). To evaluate if the LPA4/LPA-KIV9 ELISA method is affected by the size polymorphism of apo(a), the relative difference of the %bias between the results of the two assays was plotted against the size of the predominant apo(a) isoform expressed in number of KIV motifs. The bias ($r=0.043$) was not statistically significant, and the relationship between the %bias and the apo(a) KIV number ($r^2=0.002$) evidenced that the apo(a) size variation accounted for only 0.2% of the bias confirming that the LPA4/LPA-KIV9 ELISA does not appear to be

affected by the size variability of apo(a) (Fig. 3C). Based on our data, no statistically or clinically significant bias was observed between the two ELISA methods, and therefore, the methods are considered equivalent.

Comparison of the LPA4/LPA-KIV9 ELISA with the LC-MS/MS candidate reference method. Deming Regression and Bland-Altman analyses identified a mean bias of 1.17% between LPA4/LPA-KIV9 ELISA and the LC-MS/MS method with a correlation coefficient of 0.976 (Fig. 4A). The 95% confidence interval around the estimate of the %bias was from -2.180% to +4.510%, which includes 0% and is therefore not statistically significant (Fig. 4B), showing that the two methods are equivalent. The LC-MS/MS method is independent from the size variation of Lp(a) by the use of three well selected proteotypic peptides not present in KIV₂ of apo(a) (11). To determine how much of the bias in results between the two methods is explained by the size polymorphism of apo(a), we evaluated the correlation coefficient obtained between the %bias and the apo(a) isoform size. The bias ($r=0.147$) was not statistically significant, and the relationship between the %bias and the KIV number ($r^2=0.022$) evidenced that the apo(a) size variation accounted for only 2.2% of the bias confirming that the LPA4/LPA-KIV9 ELISA does not appear to be affected by the size variability of apo(a) (Fig. 4C).

Analytical performance specifications

The assay performance was validated based on the concept of total allowable error using the biological variation data extracted from the Westgard Biological Variation database (11). The recommended limit for assay imprecision for Lp(a) is 10.4%, and we have shown that the reproducibility of our assay is below this limit with a maximum CV of 9.1%. Regarding bias, the recommended performance specifications from the same database indicate a bias limit of 6.9% and the comparison of our Lp(a) results with those obtained by the "gold standard" ELISA (3) and by an LC-MS/MS candidate reference measurement procedure (12), evidenced a bias well under the 6.9% threshold. Finally, the Westgard database recommends for Lp(a) a maximum total error (TE) of 24.1%. To calculate the TE, we considered a z score equivalent to a 95% confidence interval ($z=1.65$), the maximum CV obtained during the assay reproducibility (9.1%), and either the bias against the gold standard ELISA (2.73%) or the LC-MS/MS method (1.17%). The calculated TE for the two comparisons was 17.7% and 16.2% respectively, well below the TE limit of 24.1% recommended by the Westgard database using biological variation data.

Determination of the specific peptide sequence on the epitope recognized by a-40

A peptide library spanning KIV₉ consisting of 100 overlapping peptides, each 15 amino acids in length, was designed (Fig. 5A). Antibody a-40 bound to nine

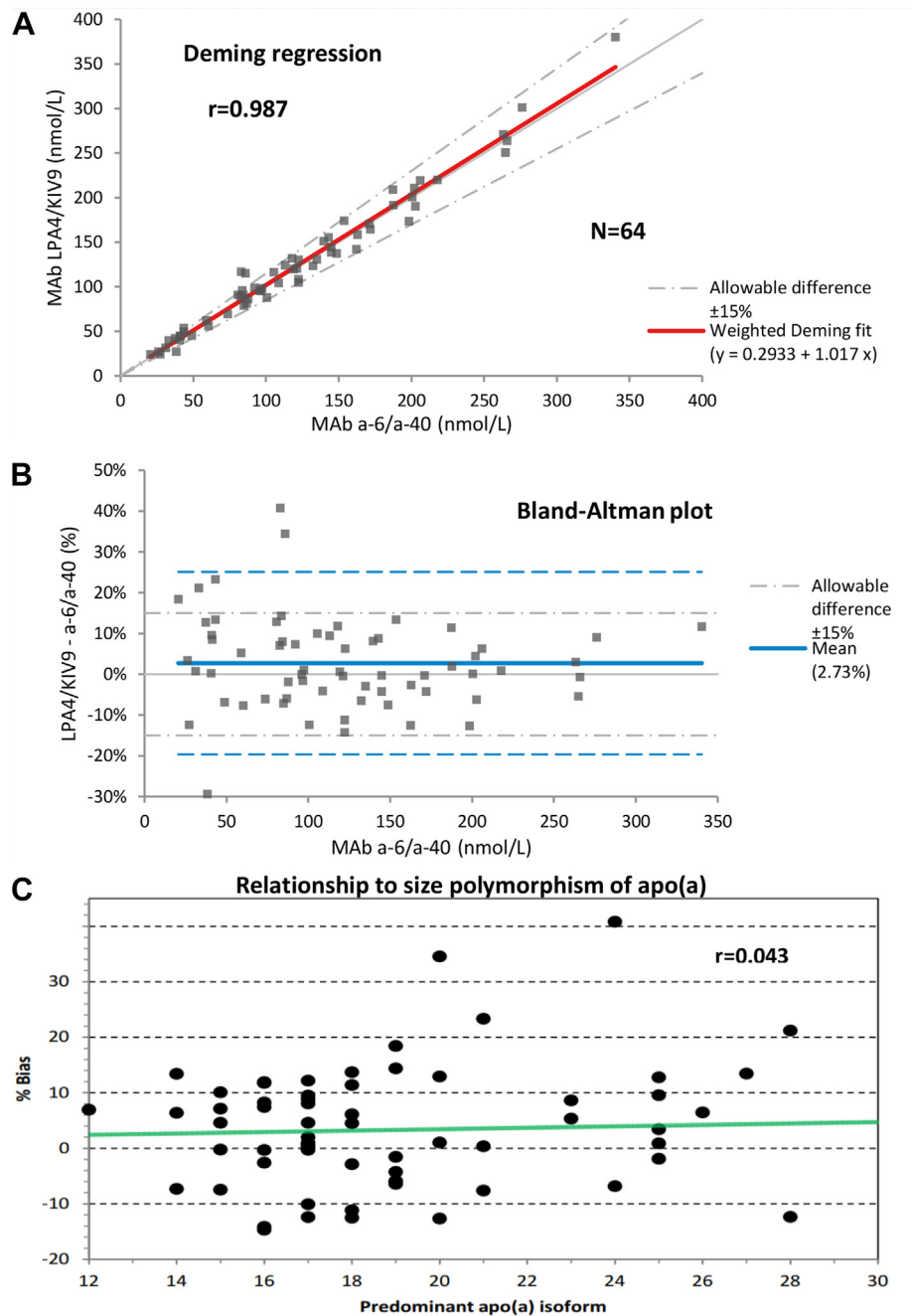


Figure 3. Comparison of LPA4/LPA-KIV9 ELISA with the a-6/a-40 ELISA as assessed by Deming regression (A), Bland-Altman plots (B), and relationship to size polymorphism of apo(a) (C).

overlapping peptide fragments on KIV₉ (Fig. 5B), which comprised the seven amino acid epitope ⁴⁰⁷⁶LETPTVV⁴⁰⁸² (Fig. 5C). This is the same sequence previously documented to be detected by monoclonal antibody LPA-KIV9. The competition experiment, showing that monoclonal antibody a-40 completely inhibits the binding of LPA-KIV9, further confirms the two monoclonal antibodies are directed to the same epitope.

DISCUSSION

This study describes the development and validation of a new, isoform-independent ELISA to measure Lp(a)

in plasma and serum. The LPA4/LPA-KIV9 ELISA was shown to be highly sensitive and linear to a broad range of Lp(a) concentrations from as low as 0.27 nmol/L to as high as 1402 nmol/L and to not be affected by the size polymorphism of apo(a). It was further shown to have no statistically or clinically significant bias relative to both the “gold standard” ELISA (5) or the LC-MS/MS candidate reference method (14) and therefore, the three methods are considered equivalent.

The development of the LPA4/LPA-KIV9 ELISA was modeled a priori after the well-validated a-6/a-40 ELISA which is the only Lp(a) method validated to be isoform independent (5). Interestingly, we have

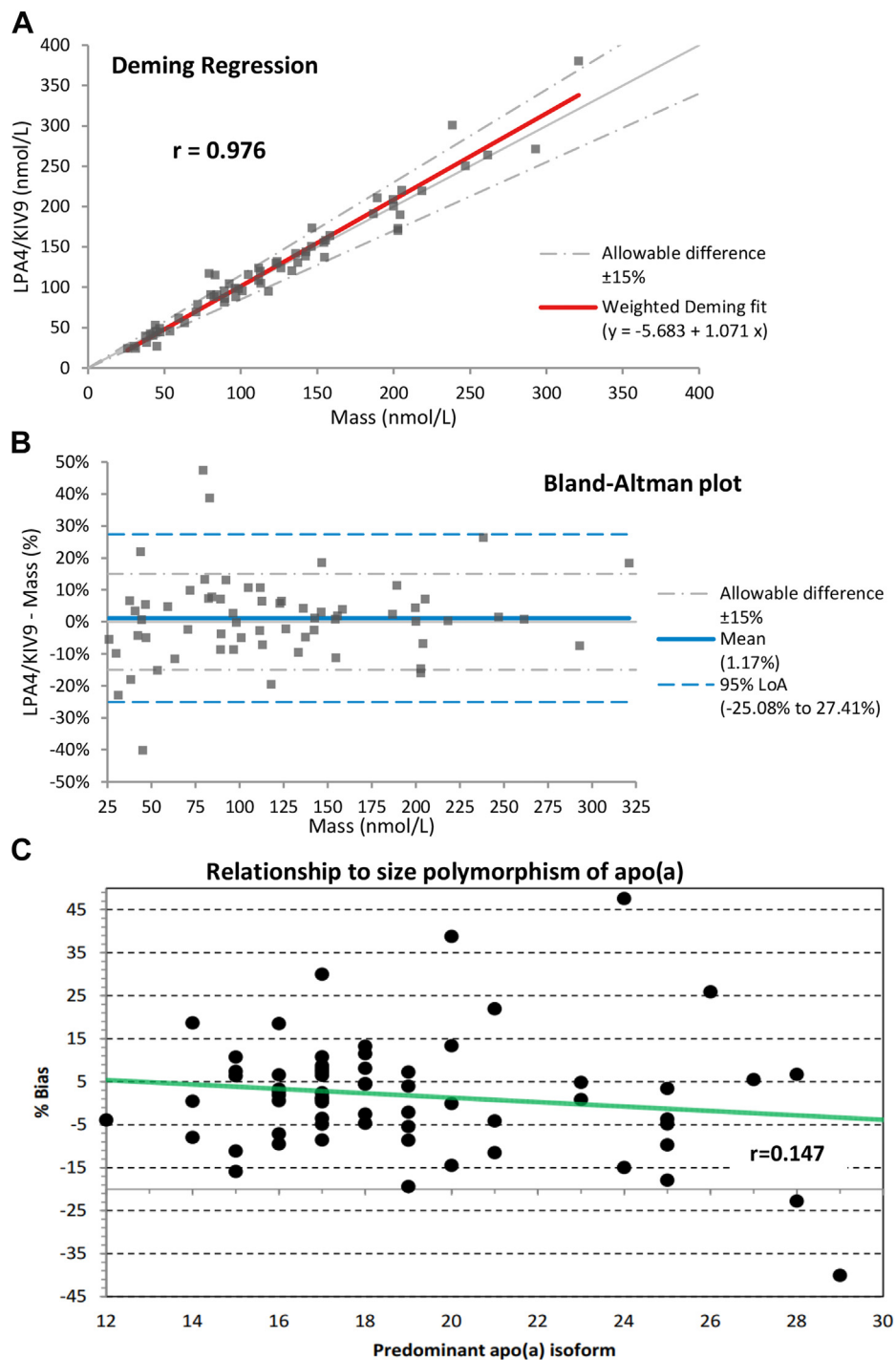


Figure 4. Comparison of LPA4/LPA-KIV9 ELISA with the LC-MS/MS method as assessed by Deming regression (A), Bland-Altman plots (B), and relationship to size polymorphism of apo(a) (C).

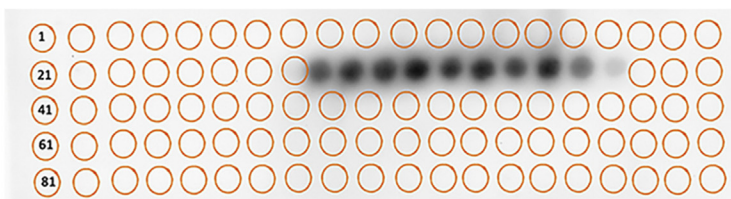
demonstrated that LPA-KIV9 and a-40 both bind to the same epitope LETPTVV on KIV₉ of apo(a). KIV₉ contains 114 amino acids (including interkringle regions), but it is highly homologous to all other kringles, except at amino acids 89–114 where modest variability exists allowing for the generation of monoclonal antibodies (4). Despite the fact that the detecting monoclonal antibodies used in the two ELISA methods are both directed to the same epitope in KIV₉, a higher than

expected bias was observed in some of the samples. In addition to possible differences in affinity, one possible explanation may relate to the fact that a-40 was generated using native, purified Lp(a) as antigen (5), whereas LPA-KIV9 was generated using a truncated apo(a) recombinant construct (6), and therefore, it may be more susceptible to conformational changes. Furthermore, the two antibodies are expected to have different sequences at the variable region as they were

A Peptide microarray sequences

1 CRNPDGKQPWCYTT	26 LTQCSETESGVLETP	51 HSEAAPEQTTPVVRQ	76 GTFSTTVTGRTCQSW
2 RNPDSGKQPWCYTTD	27 TQCSETESGVLETPPT	52 SEAAPEQTTPVVRQC	77 TFSTTVTGRTCQSW
3 NPDSGKQPWCYTTDP	28 QCSETESGVLETPPTV	53 EAAPTEQTTPVVRQCY	78 FSTTVTGRTCQSWSS
4 PDSGKQPWCYTTDPC	29 CSETESGVLETPTVV	54 AAPTEQTTPVVRQCYH	79 STTVTGRTCQSWSSM
5 DSGKQPWCYTTDPCV	30 SETESGVLETPTVVP	55 APTEQTTPVVRQCYHG	80 TTVTGRTCQSWSSMT
6 SGKQPWCYTTDPCVR	31 ETESGVLETPTVVVPV	56 PTEQTTPVVRQCYHGN	81 TVTGRTCQSWSSMTP
7 GKQPWCYTTDPCVRW	32 TEGVLETPTVVVPVP	57 TEQTTPVVRQCYHNG	82 VTGRTCQSWSSMTPH
8 KQPWCYTTDPCVRWE	33 ESGVLETPTVVVPVPS	58 EQTPVVRQCYHNGGQ	83 TGRTCQSWSSMTPHR
9 QPWCYTTDPCVRWEY	34 SGVLETPTVVVPVPSM	59 QTPVVRQCYHNGGQS	84 GRTCQSWSSMTPHRH
10 PWCYTTDPCVRWEYC	35 GVLETPTVVVPVPSME	60 TPVVRQCYHNGGQSY	85 RTCQSWSSMTPHRHQ
11 WCYTTDPCVRWEYCN	36 VLETPTVVVPVPSMEA	61 PVVRQCYHNGGQSYR	86 TCQSWSSMTPHRHQR
12 CYTTDPCVRWEYCNL	37 LETPTVVVPVPSMEAH	62 VVRQCYHNGGQSYRG	87 CQSWSSMTPHRHQRT
13 YTTDPCVRWEYCNLT	38 ETPTVVVPVPSMEAHS	63 VRQCYHNGGQSYRGT	88 QSWSSMTPHRHQRT
14 TTDPVVRWEYCNLTQ	39 TPTVVVPVPSMEAHSE	64 RQCYHNGGQSYRGTG	89 SWSMTPHRHQRTPE
15 TDPVVRWEYCNLTQC	40 PTVVVPVPSMEAHSEA	65 QCYHNGGQSYRGTFS	90 WSSMTPHRHQRTPEN
16 DPVVRWEYCNLTQCS	41 TVVVPVPSMEAHSEEA	66 CYHNGGQSYRGTFTS	91 SSMTPHRHQRTPENY
17 PCVVRWEYCNLTQCSE	42 VVPVPSMEAHSEEAAP	67 YHGNGQSYRGTFTST	92 SMTPHRHQRTPENYP
18 CVRWEYCNLTQCSETE	43 VVPVPSMEAHSEEAAPT	68 HGNGQSYRGTFTSTVT	93 MTPHRHQRTPENYPN
19 VRWEYCNLTQCSETE	44 VPVPSMEAHSEEAAPTE	69 GNGQSYRGTFTSTTVT	94 TPHRHQRTPENYPND
20 RWEYCNLTQCSETES	45 VPSMEAHSEEAAPTEQ	70 NGQSYRGTFTSTVTG	95 PPHRHQRTPENYPNDG
21 WEYCNLTQCSETESG	46 PSMEAHSEEAAPTEQT	71 GQSYRGTFTSTVTGTR	96 HRRHQRTPENYPNDGL
22 EYCNLTQCSETESGV	47 SMEAHSEEAAPTEQTP	72 QSYRGTFTSTVTGRT	97 RHQRTPENYPNDGLT
23 YCNLTQCSETESGVL	48 MEAHSEEAAPTEQTPV	73 SYRGTFTSTVTGRTC	98 HQRTPENYPNDGLTM
24 CNLTQCSETESGVLE	49 EAHSEEAAPTEQTPVV	74 YRGTFTSTVTGRTCQ	99 QRTPENYPNDGLTMN
25 NLTQCSETESGVLET	50 AHSEEAAPTEQTPVVR	75 RGTFTSTVTGRTCQS	100 RTPENYPNDGLTMNY

B Monoclonal antibody a-40 binding to 15-amino acid peptides



C Consensus sequence- LETPTVV

29 CSETESGV	LETPTVV
30 SETESGV	LETPTVVP
31 ETESGV	LETPTVVPV
32 TEGV	LETPTVVPVP
33 ESGV	LETPTVVPVPSM
34 SGV	LETPTVVPVPSM
35 GV	LETPTVVPVPSME
36 V	LETPTVVPVPSMEA
37	LETPTVVPVPSMEAH

D Competition Assay

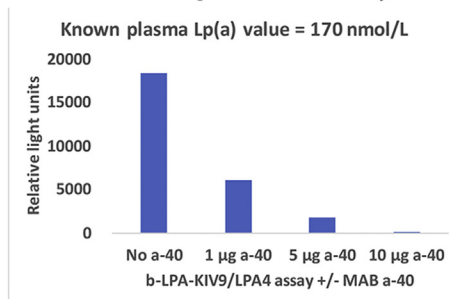


Figure 5. Determination of the specific peptide sequence on the epitope recognized by a-40. A: Displays the 15-amino acid peptide arrays spanning the entire peptide sequence of KIV9. B: Western blot of the peptide array using monoclonal antibody a-40. C: Consensus sequence LETPTVV spanning arrays 29–37 that contain the epitope. D: Competition assay demonstrating that a-40 can completely prevent LPA-KIV9 binding to Lp(a). a-40, gold-standard ELISA; Lp(a), lipoprotein(a).

generated by independent processes. However, the 2.7% bias observed between Lp(a) measurements obtained by the two ELISAs was not statistically or clinically significant, and therefore, the two methods are considered equivalent. Additionally, the apo(a) kringle IV number only accounts for 0.2% of the bias variation, confirming that the LPA-KIV9 ELISA does not appear to be affected by the size variability of apo(a). Likewise, no statistically or clinically significant bias nor apo(a) isoform bias were observed when results were compared with those obtained by the LC-MS/MS method despite the profound differences in the principle as well as assay calibration between the two methods.

It is anticipated that the determination of Lp(a) values will continue to expand with at least seven guidelines now advocating testing of Lp(a) in moderate to high-risk individuals (16). The European and Canadian guidelines have further recommended every adult

person to have Lp(a) measured at least once in their lifetime (17, 18). In clinical laboratories, analyses of Lp(a) continue to be performed by immunoassays using polyclonal antibodies against apo(a), which may bind throughout the apo(a) protein, including the variable number of the identical copies of KIV₂. These assays have the major limitation of overestimating or underestimating Lp(a) values depending on the size of apo(a) in the test samples relative to the assay calibrators. More immunocomplexes are formed between the polyclonal antibodies and the larger apo(a) isoforms, which are generally found in subjects with low plasma Lp(a) and thus the low values tend to be overestimated. In contrast, less immunocomplexes are formed with smaller isoforms, which are generally found in subjects with high plasma Lp(a), and thus, the high values tend to be underestimated (3, 8). Although optimizing the assay calibration can minimize isoform bias as

demonstrated by the Denka Seiken assay (3), which is considered the least isoform dependent method, none of the commercially available assays are truly isoform independent.


A recent study in 2020 (19) evaluated Lp(a) levels measured in the same samples by six commercially available assays showing that most bivariate correlation coefficients were greater than 0.90. However, compared to the WHO/IFCC reference material, the results of the different assays diverged from the target values by range of -8% to $+22\%$ in a concentration-dependent manner. The authors concluded that current immunological assays biases differed significantly across the clinically relevant concentration ranges in a nonlinear manner not entirely dependent on apo(a) phenotypes. Another study in 2021 from a large referral laboratory (ARUP, Salt Lake City, UT) measured Lp(a) with five commercially available assays showing significant variations when comparing results to an all-method average (20). The main limitation of both study designs is that the comparison of results was performed using Lp(a) values obtained by methods calibrated either in mg/dl of total Lp(a) mass or in nmol/L. However, both studies confirm the need of standardization of Lp(a) measurements, the traceability of the calibrators to a common reference material, and the expression of Lp(a) values in molar units.

Currently different societies have developed different thresholds for risk definition of Lp(a), such as >30 mg/dl in primary prevention settings (21), <50 mg/dl as an optimal level (22), >100 nmol/L as the 80th percentile for population levels suggested by the National Lipid Association (23), >50 mg/dl/ >125 nmol/L as a risk-modifier suggested by the ACC/AHA (24), and >70 mg/dl as entry criteria into the Lp(a) HORIZON trial (NCT04023552). With the development of potent therapies to lower Lp(a) (25–28), it will be incumbent on the Lp(a) community at large to advocate for globally standardized assays that can accurately discriminate Lp(a) risk as well as helping in defining common, specific, and accurate risk thresholds. Because the a-6/a-40 ELISA is not commercially available, the data reported here advocate for this new assay to be developed as a second, well-validated, isoform-independent ELISA that can also be a useful tool to the Lp(a) field to optimize the measurement of Lp(a) and to support global standardization of Lp(a) assays, as recently proposed (29, 30). Globally standardized assays can also harmonize the various methods to provide Lp(a) levels in molar concentrations traceable to a validated primary reference material to best define risk thresholds (3, 14, 29, 30).

In conclusion, this study demonstrates the development and validation of a new, isoform-independent ELISA to measure Lp(a) in EDTA-plasma and serum. This assay can initially be used by research laboratories for removing the confounding bias generated by differences in apo(a) isoform size. Further

refinements and/or advances will be required to use the monoclonal antibodies to develop an automated, high-throughput method that can be used in commercial laboratories.

Data availability

Data that support the plots within this publication and other findings of this study are available from the corresponding authors upon request. 

Supplemental data

This article contains [supporting information](#).

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Author contributions

S. M. M., A. G., and S. T. methodology; S. M. M., A. G., and S. T. supervision; S. M. M., A. G., S. T., N. N., and S. A., formal analysis; S. M. M., A. G., J. L. W., and S. T. writing-review and editing; N. N., S. A., investigation.

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Conflict of interest

S. M. M., N. N., and S. A. are employees of Medpace Reference Laboratories. S. M. reports consulting roles for Roche, Denka, Novartis, and research. A. G., J. L. W., and S. T. are coinventors of monoclonal antibodies directed to Lp(a) owned by UCSD directed to Lp(a). J. L. W. and S. T. receive royalties from patents on oxidation-specific antibodies and of biomarkers related to oxidized lipoproteins and Lp(a) held by UCSD. S. T. and J. L. W. are cofounders and have an equity interest in Oxitope, Inc and its affiliates (“Oxitope”) as well as in Kleanthi Diagnostics, LLC (“Kleanthi”). The terms of this arrangement have been reviewed and approved by the University of California, San Diego, in accordance with its conflict-of-interest policies. S. T. has a dual appointment at UCSD and Ionis Pharmaceuticals. J. L. W. is a consultant to Ionis Pharmaceuticals and A. G. to Kleanthi Diagnostics. All other authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

a-40, gold-standard ELISA; AMR, analytical measuring range; CV, coefficient of variation; Lp(a), lipoprotein(a); LLOQ, lower limit of quantification; QC, quality control; TE, total error; ULOQ, upper limit of quantification.

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