



**Biodemography and Social Biology** 

ISSN: 1948-5565 (Print) 1948-5573 (Online) Journal homepage: http://www.tandfonline.com/loi/hsbi20

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To cite this article: Geeta N. Eick, Paul Kowal, Tyler Barrett, Elizabeth A. Thiele & J. Josh Snodgrass (2017) Enzyme-Linked Immunoassay-Based Quantitative Measurement of Apolipoprotein B (ApoB) in Dried Blood Spots, a Biomarker of Cardiovascular Disease Risk, Biodemography and Social Biology, 63:2, 116-130, DOI: <u>10.1080/19485565.2017.1283582</u>

To link to this article: <u>http://dx.doi.org/10.1080/19485565.2017.1283582</u>



Published online: 19 May 2017.

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# Enzyme-Linked Immunoassay-Based Quantitative Measurement of Apolipoprotein B (ApoB) in Dried Blood Spots, a Biomarker of Cardiovascular Disease Risk

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#### ABSTRACT

Apolipoprotein B (ApoB) is a strong predictor of cardiovascular disease, which remains the leading cause of mortality in both higher and lower income countries. Here, we adapted an enzyme-linked immunosorbent assay (ELISA) development kit for quantitative determination of ApoB levels in serum and plasma for use with dried blood spots (DBS). After confirming the dilution linearity of the assay for DBS, we measured ApoB in 208 venous DBS samples. Then, using Passing-Bablok regression analysis and Spearman rank correlation analysis, we evaluated the correspondence in ApoB values between matched plasma and finger-prick DBS samples from 40 individuals who had ApoB values spanning the range of ApoB values observed in the 208 vDBS samples. We also evaluated assay precision and recovery, the effects of hematocrit, number of freeze-thaw cycles, and different storage temperatures on ApoB levels in DBS. There was a strong, significant correlation between plasma and DBS ApoB levels with little bias. Assay precision and recovery were within the range recommended by the U.S. government's industry guidelines for bioanalytical assay validation. The assay was not affected by the DBS matrix or physiological hematocrit levels. This DBS-based ELISA assay will facilitate population-scale assessment of cardiovascular risk in previously unexplored populations.

# Introduction

Cardiovascular disease (CVD), which includes both coronary artery disease and stroke, remains the leading cause of death worldwide in both higher and lower income countries (GBD 2013 Mortality and Causes of Death Collaborators, 2015). Although age-standardized death rates due to CVD have declined steeply since 1980 in high-income nations, rates in Eastern European, and Central, South, and East Asian countries have increased (GBD 2013 Mortality and Causes of Death Collaborators 2015; Mensah et al. 2014). Many of the economic and human costs of CVD can be mitigated through lifestyle modification,

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health promotion, and clinical appraisal; an estimated 90 percent of CVD cases are considered preventable (McGill, McMahan, and Gidding 2008; Reddy 2014).

Atherosclerosis is the primary cause of CVD and can be defined as a chronic inflammatory state caused by interactions among modified lipoproteins, monocyte-derived macrophages, T-cells, and cellular elements of the arterial wall (Glass and Witztum 2001; Ross 1999). Lipoproteins are biochemical assemblies of proteins and lipids, such as cholesterol and triglycerides, whose main function is transport of these lipids to tissues. Five lipoprotein types are recognized in humans: VLDL (very-low-density lipoprotein), IDL (intermediate-density lipoprotein), LDL, and HDL (high-density lipoprotein), all of which transport endogenous lipids; and chylomicrons, which transport dietary lipids. With the exception of HDL, of which ApoA1 is the major protein component, each of these atherogenic lipoproteins contains a single copy of apolipoprotein B (ApoB), which stabilizes the lipoprotein complex and contains recognition sites for specific receptors expressed by cells for receptor-mediated uptake. Endogenous lipids are initially transported by VLDL synthesized by the liver. After removal of triglycerides in peripheral tissue, some of the VLDL complexes are transformed to IDL or LDL particles by further removal of core triglycerides and dissociation of proteins (but not ApoB). The bulk of serum cholesterol in humans is transported by LDL. The initiator for development of atherosclerosis is thought to be modification of circulating lipoproteins in the artery wall, followed by recruitment of macrophages and their subsequent uptake of modified forms of LDL by scavenger receptors and accumulation of cholesterol esters to form foam cells. Migration of smooth muscle cells into the intimal space from the medial layer of the artery wall and subsequent inflammatory events result in the eventual formation of an atherosclerotic plaque. These plaques cause narrowing of the arteries and therefore are associated with ischemic complications, but it is rupture of the plaque itself and the resulting thrombosis that causes stroke or myocardial infarction (Glass and Witztum 2001).

Recognition of the role that circulating atherogenic particles such as VLDL, LDL, and HDL play in CVD development has resulted in use of plasma/serum levels of total cholesterol (TC) and LDL-C as markers of CVD risk in standard clinical appraisal (Contois et al. 2009). In addition, HDL-C is also routinely assessed, as high levels of this are thought to protect against CVD development (Gordon et al. 1977) and because non-HDL cholesterol (non-HDL-C), which is calculated as TC minus HDL-C to reflect the cholesterol burden not associated with HDL, has been shown to be a better marker of CVD risk than LDL-C (Lu et al. 2003; Pischon et al. 2005). However, there is now substantial recent evidence that ApoB, by itself or as the ratio of ApoB/ApoA1, is a better marker of CVD risk than TC, LDL-C, HDL-C, and various ratios of these cholesterol markers (Benn et al. 2007; Contois et al. 2009; McQueen et al. 2008). The INTERHEART study (McQueen et al. 2008) of subjects from 52 countries found that ApoB/ApoA1 was better at predicting myocardial infarction (MI) than total cholesterol and LDL cholesterol in all ethnic groups (12,461 cases and 14,637 age-matched and sex-matched controls from 52 countries). The American Diabetes Association and the American College of Cardiology issued a joint consensus statement in 2008 that ApoB should be included in the final test of the effectiveness of any LDL-cholesterol-lowering treatment (Brunzell et al. 2008). Pischon and colleagues in the Health Professional's Follow-up Study found that ApoB was a better predictor of coronary heart disease than LDL-C, and that ApoB had slightly better predictive power than non-HDL-C (Pischon et al. 2005).

The greater utility of ApoB than LDL-C as a biomarker for CVD has been attributed to the presence of only a single copy of ApoB per LDL particle. LDL particles are heterogeneous in size and content, where LDL cholesterol content can vary considerably among individuals with the same LDL particle concentration (Kwiterovich 2002). Measurement of ApoB has several other advantages. It is more amenable to standardization than approximation of a heterogeneous population of LDL particles in terms of their cholesterol content, and in particular, ApoB measurement standardization has been achieved by an International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standardization project to improve ApoB measurements (Contois et al. 2009). Almost all ApoB is bound to lipoproteins, with no measurable concentration of ApoB in aqueous medium, therefore ApoB can be used as a surrogate for LDL particle concentration. Perhaps a more important feature in the context of population-level health studies, given the complexity of interviews and sample collection and handling, is that levels of ApoB are not affected by timing of food ingestion and therefore can be measured in the nonfasted state. Non-HDL-C levels are also insensitive to fasting state, but require measurement of both total cholesterol and HDL-C.

Despite the above-mentioned advantages of using ApoB to assess CVD risk, it is not currently routinely incorporated in clinical assessments or studies. One reason for this is that it has been argued that reclassification of patients based on ApoB versus non-HDL cholesterol and/or the total/HDL cholesterol ratio is unlikely to result in a large number of treatment group or risk reclassifications overall, despite the expense of performing an additional laboratory test (Mora 2009). However, in some populations and patient sub-groups, ApoB has been convincingly demonstrated to be a better predictor of elevated cardiovascular risk than non-HDL-C (Chien et al. 2007; Sattar et al. 2004) and been shown to contribute more to the Framingham Risk Score than non-HDL-C (Sattar et al. 2004).

Several dried blood spot assays have been developed to assess ApoB levels (reviewed in McDade, Williams, and Snodgrass 2007); however, none of these studies adapted a commercially available enzyme-linked immunosorbent assay (ELISA), and these dried blood spot assays are cumbersome to run in the laboratory (Bangert, Eldridge, and Peters 1992; Dudman et al. 1985; Micic et al. 1988; Van Biervliet et al. 1982), or use antibodies produced in-house (Ohta et al. 1988) or that are no longer available (Wang, Dudman, and Wilcken 1989), or both. For these reasons, we focused on validation of a commercially available ELISA development kit (Mabtech ELISA for Human Apolipoprotein B, 3715–1H-6) for assessment of ApoB levels in dried blood spots (DBS); the paired antibodies and reagents provided in this kit have been successfully used by other researchers to assess serum levels of ApoB (Irawati et al. 2015; Jonker et al. 2010; Scalici et al. 2016).

# **Methods**

Samples Matched finger-prick DBS (fDBS), venous DBS (vDBS), and plasma samples (Eugene200 Validation Set) were collected from a convenience sample of 208 adults ( $\geq$  18 years) from the Eugene/Springfield, Oregon (USA) area between November 2014 and February 2015 as part of a validation study for the World Health Orgnization's (WHO) multicountry Study on Global Ageing and Adult Health (SAGE) biomarker assessments (Kowal et al. 2012). Age range was 18 to 77 years, and male:female (M:F) ratio was 88:120.

The Institutional Review Board at the University of Oregon approved the study protocol, and all participants gave written consent. For fDBS collection, a finger prick was made using a sterile, single-use lancet following an established protocol (McDade, Williams, and Snodgrass 2007), and up to 10 full drops of capillary whole blood were collected per participant onto up to five Whatman 903 filter paper cards. Each Whatman 903 filter paper card contains five demarcated circles for collection of up to five drops of blood per card. Only two drops of blood were collected per card (i.e., only two of five demarcated circles were used), but up to five cards were collected per person for a maximum of 10 full drops of blood per person to minimize the number of freeze-thaw cycles to which any of the cards would be subjected to during assay validation. Each drop corresponds to ~50  $\mu$ L of blood. DBS cards were dried at room temperature for 4 hours, and then stored at -80°C until analysis.

For venous blood collection, ~21 mL of blood (three 7-mL tubes) was collected by a certified phlebotomist by venipuncture into EDTA-coated vacutainer tubes. One of the three tubes was used to immediately create vDBS cards by pipetting five 50  $\mu$ L drops of whole blood onto 15 Whatman 903 filter paper cards. These were dried and stored as described for fDBS.

The second and third tubes of blood were immediately centrifuged for ~15 minutes to separate plasma. The plasma was removed and 20 100  $\mu$ L aliquots were placed in 2-mL cryotubes that were then immediately frozen at -80°C and stored until analysis. ApoB concentrations of all 208 vDBS samples were assessed.

Then, 40 individuals with vDBS ApoB values spanning the range of measured ApoB concentrations were identified, and ApoB concentrations were assessed in matched fDBS and plasma samples of these 40 individuals stored at  $-80^{\circ}$ C. The age range of this subset of individuals was 18–72 years, and the M:F ratio was 22:18. Note that we expected this subset of 40 samples to have a non-normal distribution based on their selection for ApoB concentrations across the range measured in the larger sample (n = 208).

ELISA kit The measurement range of the Mabtech Human Apolipoprotein B ELISA development kit (3715–1H-6) as reported by the manufacturer is 8–800 ng/mL. This development kit comprises four reagents: the capture monoclonal antibody mAb LDL 20/17 and the biotinylated detection monoclonal antibody LDL 11-biotin, both of which are specific for ApoB100 in its form as a VLDL/LDL–associated protein; streptavidin-horseradish peroxidase (HRP) solution; and purified LDL in 50 percent glycerol. As recommended by the manufacturer's protocol, ApoB ELISA buffer (3652-J2) was used in ELISA assays with this kit to prevent interference by heterophilic antibodies present in serum and plasma samples. Tetramethylbenzidine (TMB) was used as the substrate for HRP (TMB Microwell Peroxidase Substrate System, KPL, Cat. #50–76-00).

*DBS standards* Bio-Rad Liquichek Lipids Control Level #2 (mean ApoB concentration of 158 mg/dL based on the values reported for lot #57500 based on several analysis platforms; http://www.myeinserts.com/57500) was used to make the DBS standards as follows. Approximately 7 mL whole red blood per tube was collected by venipuncture in EDTA-coated vacutainer tubes. Tubes were centrifuged in a Plasmafuge at 1500× g for 15 minutes, the plasma and buffy coat were discarded, and then an equal volume of saline (0.86 g NaCl/ 100 mL H<sub>2</sub>O) was added, and the tubes were gently mixed for 10 minutes. This step was repeated an additional two times for a total of three washes, and the saline supernatant was discarded following the last centrifugation. Bio-Rad Level #2 was serially diluted 1:1 in ApoB

assay buffer to obtain seven ApoB concentrations (in mg/dL: 79, 39.5, 19.8, 9.9, 4.9, 2.5, 1.2). Equal volumes of these dilutions and red blood cells (RBCs) were then combined and gently mixed for 10 minutes before 50  $\mu$ L aliquots were spotted on Whatman 903 DBS cards (five aliquots/card) to make seven DBS standards with final concentrations of 39.5, 19.8, 9.9, 4.9, 2.5, 1.2, and 0.62 mg/dL. A zero DBS standard was made by combining equal volumes of washed RBCs and assay buffer. The bottom two standards (1.2 and 0.62 mg/dL) were subsequently found to be below the limit of detection of the assay, so were excluded from all calculations. DBS cards were dried for 4 hours at room temperature, and then packaged in sealed ziplock bags with desiccant and stored at  $-20^{\circ}$ C.

*Quality controls* One DBS quality control was made in the same manner described above for the DBS standards by combining equal volumes of undiluted Liquichek Lipids Control Level #1 (mean ApoB concentration of 85.3 mg/dL based on the values reported for lot #57500 based on several analysis platforms, http://www.myeinserts.com/57500) in ApoB assay buffer with packed red blood cells. The expected ApoB DBS concentration was 42.7 mg/dL. DBS quality control cards were stored at -80°C until analysis.

All standards, quality control samples, and study samples were run at least in duplicate on every assay plate.

#### Assay Protocol

DBS samples Nunc Maxisorb plates (Fisher Scientific, Cat. #12-565-135) were coated the day before the assay by aliquoting out 100 µl of mAb LDL 20/17 (1 mg/mL) diluted to a final concentration of 2  $\mu$ g/mL into the plate wells, after which the plates were placed in a refrigerator (4°C) overnight. On the day of the assay, one 3.2-mm diameter disc was punched with a handheld paper puncher (McGill MCG401CR) from the DBS standards, quality controls, and validation samples, and placed in a glass borosilicate tube containing 500 µL assay buffer and shaken for 3 hours at 250 rpm. After ~160 minutes, 500  $\mu$ L of 1 percent Triton X in phosphate-buffered saline (PBS) was added to all DBS samples (total volume in tube, 1 mL) and vortexed well to mix. Prior to loading, DBS samples and controls (but not the DBS standards) were diluted 1/10 in ApoB assay buffer. One hour before loading the plate, it was removed from the refrigerator and washed twice with 200 µL/well of PBS at pH 7.4. It was then blocked at room temperature for 1 hour by adding 200  $\mu$ L PBS + 0.05 percent Tween 20 + 0.1 percent bovine serum albumin (BSA) per well. The plate was then washed four times with  $300 \,\mu\text{L PBS} + 0.05$  percent Tween (wash buffer), and  $100 \,\mu\text{L}$  of the DBS standards, controls, or samples was added per well. Following a 1-hour incubation at room temperature (no shaking), the plate was washed four times with wash buffer, and then 100  $\mu$ L/well of a 1  $\mu$ g/mL dilution of the detection antibody (LDL 11-biotin) in assay buffer was added. The plate was allowed to incubate for 1 hour at room temperature, and then washed four times as described above. One hundred  $\mu$ L of a 1:1,000 dilution of the streptavidin-HRP provided with the kit was added per well, followed by a 1-hour incubation at room temperature. After washing four times with wash buffer, 100  $\mu$ L of room-temperature TMB substrate solution was added and the plate was incubated in the dark for 15 minutes. The reaction was stopped by adding 100  $\mu$ L of 2 M  $H_2SO_4$  to each well, and the plate was gently shaken on a plate shaker for 1 minute at ~ 300 rpm to ensure thorough mixing. Absorbance at 450 nm was then read using a spectrophotometer (BioTek ELx808), and a standard curve was constructed using four-parameter nonlinear regression as implemented in BioTek's Gen5 software. Sample and control DBS values were multiplied by a factor of 10 (diluted 10-fold more than the standard curve samples) to obtain the final ApoB concentration in mg/dL. A video of this protocol is posted on the SAGE-Eugene website (http://www.bonesandbehavior.org/sage/).

*Plasma samples* Plasma samples were treated with 1 percent Triton X in PBS by combining 10  $\mu$ L plasma with 10  $\mu$ L of 1 percent Triton X in PBS (1:2 dilution), and vortexed for ~ 5 seconds. One  $\mu$ L of this was then added to 49  $\mu$ L of ApoB assay buffer (1:2 × 1:50 = 1:100), and then 4  $\mu$ L of this 1:100 dilution was added to 236  $\mu$ L assay buffer (1:60 dilution) for a final dilution of 1:6,000. For the plasma plates, a liquid standard curve was prepared by diluting the ApoB standard supplied with the kit (125  $\mu$ g/mL purified LDL stabilized in 50 percent glycerol) in ApoB assay buffer to obtain a 1,000 ng/mL solution, which was then serially diluted 1/2 to obtain concentrations of 500, 250, 125, 62.5, 31.25, and 15.625 ng/mL. ApoB assay buffer served as the zero standard.

#### Plasma/DBS Comparisons

The relationship between plasma ApoB concentration and fDBS ApoB concentration (n = 40 matched samples) was assessed by Passing-Bablok regression analysis (Passing and Bablok 1983) using the Passing-Bablok regression tool of ACOMED Statistik (www. acomed-statistik.de), as this method takes into account uncertainty in both x- and y-values and is robust to outliers, after natural log transformation of the data (ln-transformed data had a normal distribution based on the D'Agostino-Pearson Omnibus normality test). The Spearman rank order correlation coefficient ( $R_s$ ) was also calculated as a nonparametric correlation coefficient for all DBS versus plasma comparisons using the VassarStats website for statistical computation (www.VassarStats.net). To assess the consistency of estimates based on plasma versus DBS sample types and to assess if there was any bias, we performed Bland-Altman analysis. We plotted the difference in plasma-equivalent fDBS and plasma values versus the average of these values and evaluated the bias and how many samples fell outside the 95 percent confidence intervals (Bland and Altman 1986). The correspondence between fDBS and vDBS ApoB concentrations for a subset of samples (n = 40) was assessed by Passing-Bablok regression.

#### Linearity/Parallelism

To evaluate assay parallelism, we followed the proposed guidelines and methods of Plikaytis and colleagues (1994). More specifically, five vDBS samples with moderate to high ApoB concentrations were serially diluted to 1:10, 1:20, 1:40, and 1:80, and all these samples and their serial dilutions were run on a single plate. ODmax and ODmin values (corresponding to the maximum and minimum asymptotes of the sigmoidal curve) for each sample and for the standard curve were calculated by plotting log dilution versus the OD450 value using Graphpad Prism. To linearize the sigmoidal curves for each sample and the standard curve, a fully specified (fs) logit-log model was used, where Logit (OD) fs = log (OD  $-OD_{min})/OD_{max} - OD$ ). Here,  $OD_{min}$  and  $OD_{max}$  are the lower and upper asymptotes of the sigmoidal curve (see Plikaytis et al. 1994 for a more detailed description). This logit-log model has been shown to accurately describe standard reference serum and serum sample curves that display a pronounced sigmoidal shape when plotted on an optical density (OD) versus log-dilution scale, as we observed for our DBS standard and sample curves. For each sample, a linear regression line was fitted through a plot of the log

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relative dilution of that sample versus the calculated logit (OD)fs value for each of the dilutions of that sample. This was also done for the standard curve. The dilutions of both the serially diluted samples and the standard curve samples were expressed as relative dilutions to facilitate visual comparisons using the following equation: Relative dilution<sub>i</sub> = (actual sample dilution<sub>i</sub>/maximum dilution in that series) × 100. The linearized standard curve and sample curves were then plotted on the same graph so that ApoB concentrations of each sample dilution in the series could be calculated by interpolation from the standard curve. Within-assay coefficient of variability (CV) was calculated for each sample based on the values calculated for each dilution of that series.

# Spike and Recovery

A known amount of ApoB standard provided with the kit was added to the eluate from five vDBS samples and then these samples were run in duplicate. Mean recovery was calculated as the measured ApoB concentration in the spiked sample/expected ApoB concentration based on the ApoB concentration in the unspiked vDBS sample plus the known added dose of the ApoB standard.

# Precision: Intra- and Inter-assay Coefficients of Variability (CV)

Inter-assay CV was calculated by averaging the CVs of the concentrations of the DBS control run across eight plates. Intra-assay variability was calculated by running nine duplicate wells of the DBS quality control assay in duplicate on one plate.

# Limit of Detection

The minimum detectable concentration of ApoB in DBS was calculated by adding two standard deviations to the mean optical density value of 10 DBS zero standard replicates run on a single plate and calculating the corresponding analyte concentration from the standard curve equation.

# Effect of Hematocrit

To evaluate the effect of hematocrit on assay performance, four 7-mL EDTA-coated vacutainers of whole blood (~ 7 mL of blood per tube) were centrifuged at 1520 g in a Horizon Plasmafuge for 15 minutes to obtain packed red blood cells (RBCs; equivalent to a hematocrit of 100 percent), and the plasma was reserved. RBCs were then diluted with the reserved plasma to obtain samples with physiologically relevant hematocrit values of 30, 40, 50, and 60 percent and spotted onto filter paper. The sample with 50 percent hematocrit was assumed to be typical of most DBS, and the values obtained for the other percentage hematocrit DBS were assayed and expressed as the percentage recovery of the concentration in the 50 percent hematocrit sample (100  $\times$  observed/expected).

# Analyte Stability

To assess the temperature stability of ApoB in DBS, venous DBS cards from one individual with a moderately high ApoB level (82 mg/dL) were stored at 22°C (controlled ambient), 37°C (hot ambient), and -20°C (frozen) for 2, 7, 14, and 21 days after collection and then transferred to -80°C. Percentage recovery was calculated relative to the ApoB level in the sample stored at -80°C immediately after collection after 4 hours of drying and that was thawed only once for the assay. To assess the freeze-thaw stability of ApoB in

DBS, venous DBS cards from one individual with a moderately high ApoB level (77 mg/ dL) were exposed to 2, 3, 5, 9, and 15 freeze-thaw cycles ( $22^{\circ}$ C for 8 hours then  $-80^{\circ}$ C for 16 hours) after being stored at  $-80^{\circ}$ C after 4 hours of drying. Cards were then stored at  $-80^{\circ}$ C and assayed in a single batch along with a DBS card from the same individual that had not previously been thawed. Percentage recovery was calculated relative to the ApoB concentration in the sample that had been thawed only once for this experiment.

#### Results

#### Plasma/DBS Comparisons

The Passing-Bablok regression equation for the plot of log-transformed fDBS and plasma ApoB concentrations was y = 1.35x - 1.30 (Figure 1a), and there was a strong correlation between fDBS and plasma ApoB concentrations ( $R_s = 0.82$ , p < 0.01). The Passing-Bablok regression equation for the plot of log-transformed vDBS versus plasma ApoB concentrations was similar to that for the fDBS versus plasma ApoB concentrations, y = 1.03x + 1.13, and for the fDBS, there was a very strong correlation between vDBS and plasma ApoB values ( $R_s = 0.83$ , p < 0.01; not shown). Prior to Bland-Altman analysis of fDBS versus plasma values, fDBS values were converted into plasma-equivalent fDBS values using the Passing-Bablok regression equation. Bland-Altman analysis of the difference in plasmaequivalent fDBS and plasma values versus the average of these values indicated the presence of a small bias (1.36), but only 1 of 40 values fell outside the 95 percent limits of agreement (Figure 1b). The Passing-Bablok regression equation relating fDBS and vDBS values for a subset of samples (n = 40) was y = 0.81x + 0.22 (Figure 1c), and the slope value of 1 was within the 95 percent confidence interval (CI) of the linear slope (slope = 0.81, 95 percent CI [0.61, 1.07]), indicating no proportional difference between fDBS and vDBS ApoB values. There was a strong correlation between vDBS and fDBS values ( $R_s = 0.76$ , p < 0.01).

*Linearity/parallelism* Within-assay CVs of the serially diluted DBS dilutions of the five vDBS samples ranged from 1.67 to 16.9 percent (average, 8.8 percent) (Table 1). While a decrease in concentration with increased dilution was noted for four of the five samples, this decrease was well within acceptable limits ( $\leq$  20 percent) (Plikaytis et al. 1994).

*Quality controls* Average percentage recovery of ApoB from the DBS control (expected DBS value, 42.7 mg/dL; measured DBS values for eight plates,  $37.3 \pm 2.8 \text{ mg/dL}$  [SD]; plasma equivalent value, 147.9 mg/dL) was 87 percent (range, 75–101 percent).

*Spike and recovery* In the spike and recovery experiment, percentage recovery ranged from 88 to 104 percent, indicating that the DBS matrix is valid for the assay procedure (U. S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, and Center for Veterinary Medicine, 2013).

*Precision* The intra-assay CV was 6.2 percent, while the inter-assay CV was 7.6 percent, which are within the recommended limits for ELISA assays (U.S. Department of Health and Human Services et al. 2013).

Limit of detection The lower limit of detection of the DBS assay was 1.65 mg/dL.

*Effect of hematocrit* Hematocrit did not appear to have a drastic effect on sample recovery, with percentage recovery (relative to the level in the 50 percent hematocrit sample) ranging from 100 to 126 percent (mean, 114 percent).

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**Figure 1.** (a) Passing-Bablok regression plot of finger-prick DBS (fDBS) ApoB concentration versus plasma values for 40 matched samples after natural log transformation of ApoB values. The equation to convert fDBS values into plasma equivalents is indicated above the graph. (b) Bland-Altman plot of the difference in plasma and plasma-equivalent fDBS ApoB values vs. the average of these values (n = 40). Bias was negligible (1.4), and only one point fell outside the 95 percent confidence intervals indicated by the dotted lines, indicating no significant bias in ApoB measured in plasma vs. ApoB measured in fDBS. (c) Passing-Bablok regression plot of venous DBS vs. fDBS ApoB concentrations. The value of 1 fell within the 95 percent confidence intervals of the slope (0.6–1.1), indicating no systematic proportional difference between ApoB values in venous and capillary blood. The line of equivalence is drawn for reference (dotted line), and the Passing-Bablok regression equation is provided above the graph.

Analyte stability A noticeable decrease in ApoB levels was seen in DBS cards stored at room temperature for more than 14 days before freezing at  $-80^{\circ}$ C, and this decrease became more marked over time. There was a linear decrease with time in measured ApoB concentration in DBS cards stored at 37°C before freezing. Storage at  $-20^{\circ}$ C for 21 days before freezing at  $-80^{\circ}$ C had no marked effect on measured ApoB levels (Figure 2a). Furthermore, there was a sustained decrease in measured ApoB levels starting after five freeze-thaw cycles (Figure 2b).

#### Discussion

Apolipoprotein B (ApoB), the main protein component of low-density lipoprotein (LDL) particles, has been demonstrated to be a powerful predictor of cardiovascular risk. Numerous studies have shown ApoB to be a better predictor of cardiovascular events than LDL-C or total cholesterol (Gotto et al. 2000; Pischon et al. 2005; Walldius, Aastveit,

vDBS		ApoB concentration interpolated from DBS standard	Mean ApoB	Standard	CV
Sample	Dilution	curve*	[mg/dL]	deviation	(%)
#1	1:10	186.47			
	1:20	185.09			
	1:40	185.07			
	1:80	179.55	184.04	3.07	1.67
#2	1:10	226.14			
	1:20	218.79			
	1:40	207.30			
	1:80	181.34	208.39	19.63	9.42
#3	1:10	197.50			
	1:20	190.53			
	1:40	175.86			
	1:80	134.74	174.66	28.10	16.09
	1 1 0	100 50			
#4	1:10	189.52			
	1:20	1/9.02			
	1:40	166.81			
	1:80	134.83	167.54	23.70	14.15
#5	1.10	197 50			
#5	1.10	204.20			
	1.20	207.37			
	1:40	200.33	201.00		2 70
	1:80	197.50	201.98	5.45	2.70

**Table 1.** Assessment of the parallelism of the ApoB DBS assay by calculation of intra-assay concentrations of five serially diluted vDBS samples. All CV values were < 20 percent, indicating acceptable assay parallelism according to Plikaytis et al. (1994).

Note. \*Described in detail in Plikaytis et al. (1994).



**Figure 2.** (a) Effect of different temperature storage conditions on ApoB concentration in DBS. Data are expressed as the percentage recovery relative to the titer measured for the DBS card stored at  $-80^{\circ}$ C after collection and 4 hours drying at room temperature (RT). DBS cards were kept at 37°C (filled diamonds), room temperature (RT, ~ 22°C, gray squares), or  $-20^{\circ}$ C (open triangles), for 2, 7, 14, and 21 days. Error bars indicate SEM. Note that all cards were from a single donor. (b) Effect of number of freeze-thaw cycles on measured ApoB concentrations in DBS from a single donor. Data are expressed as the percentage recovery relative to the concentration measured for a DBS card stored at  $-80^{\circ}$ C after collection and 4 hours drying at room temperature (RT) that was thawed only once for this assay. Error bars indicate SEM.

and Jungner 2006). Furthermore, ApoB levels can be an advantageous method for stratifying vascular disease risk in people with diabetes (Williams et al. 2003). ApoB has several advantages over traditional cholesterol measures, including: (1) it is a direct

measure (LDL-C is calculated) (Barter et al. 2006), (2) it can be measured from blood in a nonfasting state (Sundvall et al. 2011), (3) it can be more reliably measured in DBS than total cholesterol (Crimmins et al. 2014), and (4) it predicts cardiovascular risk in individuals who have therapeutically lowered LDL (Contois et al. 2009). Measurement of ApoB in DBS also makes ApoB a feasible CVD risk marker for large population health studies like WHO SAGE or the U.S Health and Retirement Study.

In this study, we developed and validated a dried blood spot-based ELISA for ApoB. As for any analyte, the analysis of ApoB levels in DBS can reduce the burden on participants, facilitate population-scale research, and increase convenience for researchers due to simplified sample collection procedures, storage, and transportation (Li and Lee 2014; McDade, Williams, and Snodgrass 2007; Mei et al. 2001). We noted in McDade, Williams, and Snodgrass (2007) the availability of several DBS assays for the measurement of ApoB; however, none of these assays has been widely used for a variety of reasons, ranging from outdated methods to unavailable assay components. We focused on adapting the ApoB ELISA development kit from Mabtech for use with DBS rather than its human ApoB ELISA<sup>PRO</sup> kit, which comes with precoated plates and all the reagents and solutions required to quantify ApoB in solution, because of significant concerns associated with the use of commercial ELISA kits, namely their limited shelf life and often mercurial commercial life. Of the four components in the detection kit, only three are used in the DBS-based assay (capture antibody, biotinylated detection antibody, and streptavidin-HRP). Both the capture and detection antibodies can be stored at  $-20^{\circ}$ C after aliquoting to minimize the number of freeze-thaw cycles in a frozen state without loss of activity (Sigma-Aldrich 2015), while streptavidin-HRP is widely available from a number of commercial suppliers, although the exact dilution used in the assay would have to be determined using a test plate. The calibrators used to make the DBS standards and controls (Bio-Rad Liquichek Lipid and Immunology controls) are human-assayed protein controls used to monitor the precision of testing procedures in clinical laboratories and are therefore expected to have commercial longevity.

Major limitations of our study are that we did not quantify ApoB levels in plasma using the gold standard reference method of immunoturbidimetry (Contois et al. 1996) and we did not include liquid QC samples on the plates on which we ran the plasma samples. However, according to the manufacturer of the Mabtech ApoB development kit, it has been validated for quantitative measurement of ApoB100 in human plasma and serum samples in its form as a VLDL/LDL-associated protein. Furthermore, the good correspondence between the level of ApoB measured in the DBS quality control and the expected value, taken together with the average and range of ApoB in the fDBS samples (average, 118.3 mg/dL, range, 39–213 mg/dL after conversion to plasma equivalent values using the Passing-Bablok regression equation) being largely consistent with what has previously been reported in other, much larger population-based studies of CVD-asymptomatic individuals (Gotto et al. 2000: average, 122 mg/dL; Jungner et al. 1998: average, 126 mg/ dL; 5th percentile, 76 mg/dL; 95th percentile, 195 mg/dL; Walldius, Aastveit, and Jungner 2006: average, 126 mg/dL) gives us confidence that the DBS version of this assay provides a reasonable, if not clinically precise, estimate of circulating ApoB levels.

It should also be noted that we developed this assay for population-based scientific research rather than clinical diagnosis; the linear relationship between plasma and fDBS ApoB levels and the normal distribution of ApoB values within our validation population

suggest that relative comparison of ApoB levels in DBS within and among populations using this assay will yield useful information. Previous studies that analyzed populationlevel ApoB data have reported a general increase in ApoB level with age (Benn et al. 2007) and differences between males and females (Benn et al. 2007; McQueen et al. 2008). However, these studies were performed in high-income nations, so it is unclear whether these patterns are innate and universal.

In summary, we developed and validated an ELISA assay to measure levels of ApoB in dried blood spots. We confirmed acceptable parallelism of the assay with respect to DBS and found a linear relationship between DBS and plasma ApoB levels. Inter- and intra-assay variations were within the accepted range for ELISA assays, and ApoB levels appeared to be stable in DBS stored at  $-20^{\circ}$ C for an extended period of time, although we recommend limiting the number of freeze-thaw cycles to fewer than five as ApoB stability does appear to decrease with the number of freeze-thaw cycles. The dramatic decrease in ApoB concentrations observed after storage at 37°C and prolonged storage at room temperature also emphasizes the need for researchers to develop collection and shipping protocols that will minimize exposure to elevated temperature and/or prolonged exposure to moderate temperatures. Hematocrit did not appear to hinder measurement of ApoB from DBS. Together, these findings indicate that the DBS-based ELISA assay for ApoB described here will provide a useful tool for assessment of CVD risk in population-based studies.

#### Acknowledgments

We would like to acknowledge the help and enthusiasm of the Eugene200 team of volunteer staff who made this study possible (Tyler Barrett, Blanche Blumenthal, Robyn Brigham, Haley Brown, Micaela Burns, Zach Clayton, Devan Compton, Tyler Fording, Theresa Gildner, Elisabeth Goldman, Anna Hanson, Melissa Liebert, Colin Lipps, Brian McCree, Sophie McGinley, Lauren Moore, Caroline Porter, Josh Schrock, Molly Turner, and Oliver Wald), Kirstin Sterner and Nelson Ting for access to their lab facilities, and the Eugene200 participants for contributing samples. We also appreciate the rigorous and in-depth review of our manuscript; it benefitted considerably from the reviewers' comments.

# Funding

Funding was provided by a University of Oregon Richard A. Bray Faculty Fellowship, NIH NIA Interagency Agreement YA1323-08-CN-0020, and NIH R01-AG034479.

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