Eick Geeta (Orcid ID: 0000-0001-7512-3265)

A Dried Blood Spot-Based Method to Measure Levels of Tartrate-Resistant Acid Phosphatase 5b (TRACP-5b), A Marker of Bone Resorption

Running title: DBS Measurement of TRACP-5b

Geeta N. Eick[1], Maureen J. Devlin[2], Tara J. Cepon-Robins[3], Paul Kowal[1,4,5], Lawrence S. Sugiyama[1], and J. Josh Snodgrass[1]

[1]Department of Anthropology, University of Oregon

[2]Department of Anthropology, University of Michigan

[3]Department of Anthropology, University of Colorado, Colorado Springs

[4]World Health Organization (Switzerland)

[5]University of Newcastle Research Centre for Generational Health and Ageing (Australia)

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Correspondence
Geeta N. Eick
Global Health Biomarker Laboratory
Department of Anthropology
University of Oregon
Eugene, OR 97403
E-mail: geeta.eick@gmail.com

ABSTRACT

Objectives: A number of basic questions about bone biology have not been answered, including population differences in bone turnover. In part, this stems from the lack of validated minimally invasive biomarker techniques to measure bone formation and resorption in field-based population-level research. The present study addresses this gap by validating a fingerprick dried blood spot (fDBS) assay for tartrate-resistant acid phosphatase 5b (TRACP-5b), a well-defined biomarker of bone resorption and osteoclast number.

Methods: We adapted a commercially available enzyme-linked immunosorbent assay (ELISA) kit from MyBiosource for the quantitative determination of TRACP-5b levels in serum and plasma for use with DBS. We used a rigorous process of assay modification and validation, including the use of a matched set of 189 adult plasma, fDBS, and venous DBS (vDBS) samples; parameters evaluated included precision, reliability, and analyte stability.

Results: Plasma and DBS TRACP-5b concentrations showed a linear relationship. There were no systematic differences in TRACP-5b levels in fDBS and vDBS, indicating no significant differences in TRACP-5b distribution between capillary and venous blood. Parallelism and spike-and-recovery results indicated that matrix factors in DBS do not interfere with measurement of TRACP-5b levels from DBS using the validated kit. Intra- and inter-assay CVs were 5.0% and 12.1%, respectively. DBS samples should preferably be stored frozen but controlled room temperature storage for up to a month may be acceptable.

Conclusions: This DBS-based ELISA assay adds to the methodological toolkit available to human biologists and will facilitate research on bone turnover in population studies.

(Words: 249 [250 as limit])

Key words: human skeletal biology; bone turnover; osteoporosis; biomarker; immunoassay

1. INTRODUCTION

Bone, which comprises mineralized connective tissue, is a highly dynamic organ that is continually undergoing remodeling, with bone resorption by osteoclasts and bone formation by osteoblasts (Sims & Martin, 2014). The balance between bone formation and resorption changes throughout life in ways that can be informative about life history events, providing a window into the lived experience of the individual since bone turnover is shaped not only by genetics but also mechanical forces, dietary factors, and other aspects of lifestyle and environment. There is, therefore, broad interest in biological anthropology in uncovering the specific factors that influence bone turnover throughout the life course.

A number of basic questions about bone biology have not yet been answered despite steady progress in the epidemiology and treatment of osteoporosis (Bartl & Frisch, 2009; Madimenos, Liebert, Cepon-Robins, Snodgrass, & Sugiyama, 2015; Marcus, Dempster, Cauley, & Feldman, 2013). This lack of insight into the factors that affect bone metabolism stems from a narrow focus on clinical settings in the context of wealthy nations, as well as the lack of field-friendly techniques to measure biomarkers of bone formation and resorption. The impact of the availability of these technologies is exemplified by the development of portable calcaneal ultrasonometers to measure bone mineral density (BMD); use of these ultrasonometers allowed researchers to obtain high-quality data on bone health in rural, isolated populations, namely indigenous Shuar and non-indigenous *Colono* from Ecuador (Madimenos et al., 2011; Madimenos et al., 2015) and the Tsimane of Bolivia (Stieglitz et al., 2015; Stieglitz, Trumble, Kaplan, & Gurven, 2017).

There are currently no validated minimally invasive techniques for the measurement of biomarkers of bone formation and resorption that can be used in samples collected in field settings (i.e., outside of a clinical laboratory environment). Although saliva and urine samples have been used to access certain biomarkers (most notably cortisol and testosterone, where multiple daily samples are required to document diurnal fluctuations), blood is the biological substance of choice or necessity for many analytes. In particular, capillary whole blood from a finger prick collected onto filter paper (dried blood spots [DBS]) has opened a realm of possibilities, and the last decade has seen the adoption of this method in biological anthropology, epidemiology, psychology, and a variety of other disciplines (Demirev, 2013; Li & Lee, 2014; McDade, Williams, & Snodgrass, 2007; Mei, Alexander, Adam, & Hannon, 2001). Advantages of using DBS include simplicity of collection, ease of transport, generally good analyte stability, decreased biohazard risk, and low cost (Freeman et al., 2018; McDade et al., 2007).

Tartrate-resistant acid phosphatase 5b (TRACP-5b) is a well-defined biomarker of bone resorption and osteoclast number encoded by a single gene in humans. TRACP-5 is expressed in both myeloid lineage cells (e.g., macrophages) and in osteoclasts, but has different secondary modifications in the two cell types. TRACP-5 produced in myeloid cells is modified with sialic acid and contains high-mannose-type sugar chains (TRACP-5a), while TRACP-5 produced by osteoclasts is not modified with sialic acid and has multi-antennary complex-type sugar chains (TRACP-5b) (Kawaguchi et al., 2008; Ohashi et al., 2006; Ohashi et al., 2007). In addition, these two enzymes have different pH optima of 5.2 and 5.8, respectively. These biochemical differences are also reflected in the differences in phosphatase activity of the two enzymes, with TRACP-5b displaying a higher specific activity than TRACP-5a (Janckila, Takahashi, Sun, & Yam, 2001), although the biological functions of tartrate-resistant acid phosphatases

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are still unknown (Janckila & Yam, 2009). Circulating levels of TRACP-5b correlate with the number of osteoclasts (Janckila & Yam, 2009) and TRACP-5b is therefore widely used as a bone metabolic marker of osteoclast-mediated bone resorption (Chu, Chao, Lin, Janckila, & Yam, 2003; Halleen et al., 2001; Halleen et al., 2002; Hannon et al., 2004; Nenonen et al., 2005).

In the present study, our goal was to validate a commercially available ELISA kit (MyBiosource, MBS704711) for assessment of TRACP-5b levels in DBS as a proxy for osteoclast activity. This research opens the door to investigating population differences in bone acquisition, turnover, and the effects of factors such as senescence, nutrition, disease, exercise, pregnancy, and lactation on bone mass.

2. METHODS

The present study was performed in the Global Health Biomarker Laboratory at the University of Oregon and used a rigorous and systematic process of evaluation, modification, and validation. All data are available upon request to the first author.

ELISA kit. We validated the Human Tartrate-Resistant Acid Phosphatase 5b ELISA kit (MyBiosource, MBS704711), a quantitative colorimetric sandwich ELISA developed to assay TRACP-5b levels in serum, plasma, and tissue homogenates. The measurement range of the kit as reported by the manufacturer is 0.312 IU/L – 20 IU/L, with a reported lower limit of detection (LOD) for TRACP-5b of 0.078 IU/L. Reported TRACP-5b levels in the serum of normal, evidently healthy adults of both sexes range from 1.89 ± 0.81 IU/L, n = 32 (Mokhtar et al., 2017); 2.07 ± 0.83, n = 33 (Tang et al., 2017); and 1.2 - 4.2 IU/L in young adults (Nishizawa et al., 2013).

Samples. Validation samples comprised matched fingerprick DBS (fDBS), venous DBS (vDBS), and plasma samples ('Eugene200 Validation Set') collected from a convenience sample of 189 adults (≥18 years) from the Eugene/Springfield, Oregon (USA) area between November 2014 and February 2015 as described in detail previously (Eick, Kowal, Barrett, Thiele, & Snodgrass, 2017). All samples were collected in the evening between 5 pm and 9 pm, but exact time of collection was not recorded and, therefore, not considered in the analyses.

IRB approval for this study was obtained from the Committee for the Protection of Human Subjects, University of Oregon (protocol # 7062016.007) and informed consent was obtained from all participants.

Detection of TRACP-5b in dried blood spots. As a first step to evaluate if TRACP-5b can be detected in DBS, two different quality control (QC) DBS sets were created. The first set of QC DBS was created by spiking 1 mL of whole blood (WB) collected by venipuncture with 100 μl of 40 IU/L of TRACP-5b obtained by resuspending the lyophilized standard provided with the kit in 500 μl assay buffer and then pipetting out 60 μl of this mixture onto Whatman 903 filter paper cards. Cards were then air dried on the bench for 4 hours and then double bagged in sealed plastic bags, each containing desiccant, and frozen at -28°C. The second set of quality controls was created by collecting approximately 7 mL whole blood per tube by venipuncture in EDTA-coated Vacutainer tubes and centrifuging the tubes in a Plasmafuge at 1500x g for 15 minutes. Plasma and buffy coat were subsequently discarded, and then an equal volume of saline (0.86 g NaCl/100 mL H_2O) 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

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supernatant was discarded following the last centrifugation. An equal volume of washed red blood cells obtained in this manner was spiked with sample diluent from the kit containing 40 IU/L of TRACP-5b, and then 60 μl of this mixture was pipetted onto DBS cards to create RBC QCs. Either one or two 6 mm punches from these QCs, as well as WB and RBC zero controls (unspiked WB and RBCs mixed with an equal volume of sample diluent with no TRACP-5b added, respectively), were eluted in 180 μl of sample diluent overnight at 4°C.

TRACP-5b levels in these eluants were assessed using the MyBiosource TRACP-5b ELISA kit with all steps performed according to the manufacturer's protocol. Color was allowed to develop for 20 minutes (manufacturer recommends 15-30 min). Absorbance at 450 nm was read using a spectrophotometer (BioTek ELx808) and a standard curve was constructed using 4-parameter nonlinear regression as implemented in BioTek's Gen5 software.

Measurement of TRACP-5b in E2V2 samples. TRACP-5b concentrations were assessed in 189 vDBS samples (stored at -80°C; M:F 80:109) by eluting two 6-mm punches in 180 μl sample eluent and loading 100 μl of this per well. After plotting a histogram of the distribution of TRACP-5b levels, 49 individuals with vDBS TRACP-5b values spanning the range of measured TRACP-5b concentrations were identified, and TRACP-5b concentrations were assessed in matched fDBS and undiluted plasma samples of these 49 individuals, which were stored at -80°C.

Plasma/DBS comparisons. The relationships between plasma, fDBS, and vDBS TRACP-5b concentrations in samples with a TRACP-5b higher than the LOD (1.1 IU/L) were assessed by Passing-Bablok regression analysis as this method takes into account uncertainty in both x- and y-values and is robust to outliers.

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 by plotting the difference in plasma-equivalent fDBS values (transformed using the appropriate Passing-Bablok regression equation) and plasma values versus the average of these values, and evaluated the bias and how many samples fell outside the 95% confidence intervals (Bland & Altman, 1986).

Parallelism. Four vDBS samples with a high endogenous concentration of TRACP-5b were serially two-fold diluted to obtain 1/2, 1/4, and 1/8 dilutions. Only two of the four samples had a sufficiently high original concentration to be able to obtain dilutions that were within the assay range. For these samples, the in-range measurements were multiplied to obtain the equivalent 'neat' concentration, and the CV of these concentrations was then calculated.

Spike & recovery. Six 6-mm punches from six different individuals were pooled and eluted in 600 μl of sample diluent. The 'neat' eluent, as well as ½ and ¼ dilutions of this eluent were then spiked with 8 IU/L, 4 IU/L, 2 IU/L, or 0 IU/L of TRACP-5b standard provided with the kit and run in duplicate (total of 24 wells). Mean recovery was calculated as the measured TRACP-5b concentration in the spiked sample/expected TRACP-5b concentration based on the TRACP-5b concentration in the unspiked vDBS sample plus the known added dose of the TRACP-5b standard.

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Precision: Intra- and inter-assay coefficients of variation (CV). Inter-assay CV was calculated by averaging the CVs of the concentrations of high and low DBS controls across six plates. These DBS controls were made by spiking 1 mL of whole blood with 40 IU/L (high DBS control) or 0 IU/L TRACP-5b (low DBS control; only endogenous TRACP-5b being measured). Intra-assay variability was calculated by running eight duplicate wells each of the high and low DBS quality controls on one plate and calculating the average of the high and low DBS CVs.

Limit of detection. The minimum detectable concentration (limit of detection [LOD]) of TRACP-5b in DBS was calculated by adding two standard deviations to the mean optical density value of 14 wells containing assay buffer only and calculating the corresponding analyte concentration from the standard curve equation.

Analyte stability. Venous DBS cards from one individual with a moderate TRACP-5b level (2.27 IU/L) were stored at 22°C (controlled ambient), 37°C (hot ambient), and -28°C (frozen) for 2, 7, 14, and 21 days after collection and then transferred to -80°C. Percentage recovery was calculated relative to the titer in the sample stored at -80°C immediately after collection and dried and thawed only for the assay.

3. RESULTS

Detection of TRACP-5b in dried blood spots. TRACP-5b levels were consistently higher in the spiked DBS samples than the matched unspiked samples (Wilcoxon's signed rank test, n = 12, W = 0, p < 0.05 one-tailed), indicating that TRACP-5b can be measured from DBS. As expected, use of two 6-mm punches yielded higher absorbance (and, therefore, concentration) values than use of a single 6-mm punch, and the absorbance values obtained when two punches were used were always greater than that of the lowest standard (0.312 IU/L), whereas this was not always the case for the single punches. Therefore, two 6-mm punches were used in all subsequent assays.

Measurement of TRACP-5b in E2V2 samples. The range of TRACP-5b levels measured in the vDBS after removal of samples with values below the LOD (n = 6 samples had a TRACP level ≤ 1.1 IU/L; see below) was 1.12 – 5.58 IU/L, with a median value of 2.06 IU/L and a mean of 2.28 IU/L. The range of TRACP-5b concentrations in the 36 fDBS samples (after conversion to plasma equivalents) ranged from 0.83 – 4.67 IU/L, with a mean concentration of 2.29 IU/L and median concentration of 1.86 IU/L.

Plasma/DBS comparisons. There was a clear linear relationship between levels of TRACP-5b in pairwise comparisons of the various sample types, as shown in the plots presented in **Fig. 1**.

There was negligible bias in the fDBS vs. plasma Bland-Altman plot (bias = 0.06, 1 sample outside the 95% CI; **Fig. 2A**), indicating that TRACP-5b levels measured in plasma and fDBS are not systematically different. There was slightly more bias in the vDBS vs. fDBS comparison (bias = 0.35, 3 samples outside the 95% CI), with vDBS values generally slightly higher than fDBS values (**Fig. 2B**). This bias is likely due to

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the generally smaller spot diameters of the fDBS than the vDBS, with corresponding lower analyte concentrations in punches taken from the fDBS than the vDBS (George & Moat, 2016).

Parallelism. The average CV of the two serially diluted samples was 30%, which is considered acceptable in commercial biomarker development laboratories (CV ≤ 30%) (Stevenson & Purushothama, 2014).

Spike & recovery. Percentage recovery ranged from 91% to 134%, with a mean of 112.25%, indicating that the DBS matrix is a valid sample matrix that does not contain factors that interfere with TRACP-5b measurement using the MyBiosource ELISA kit described here.

Precision: Intra- and inter-assay coefficients of variation (CV). The intra-assay CV was 5.0%, and the inter-assay CV was 12.1%.

Limit of detection. The LOD of this assay as performed in our lab was 1.1 IU/L.

Analyte stability. Average percentage recovery ranged from 93-116% (mean, 101%) for samples stored at -28°C for 2-28 days, 101-108% (mean, 104%) for samples stored at controlled room temperature for 2-28 days, and 119-140% (mean, 131%) for samples stored at 37°C for 2-28 days. No trend according to number of days stored was evident for any of the storage temperatures.

4. DISCUSSION

Using a rigorous approach of evaluation, modification, and validation, we adapted an existing commercial ELISA that specifically measures levels of TRACP-5b, a protein marker of osteoclast activity and therefore bone resorption, for use with DBS. Levels of TRACP-5b measured in fDBS were consistent with those reported previously for serum/plasma samples, with a mean plasma-converted TRACP-5b level of 2.29 ± 1.1 IU/L. In addition, TRACP-5b levels were higher in males than females, as expected (mean plasma-equivalent fDBS values: 2.59 ± 1.3 [SD] in males versus 2.07 ± 1.0 [SD] in females). TRACP-5b levels in fDBS and vDBS were comparable, indicating no significant differences in TRACP-5b distribution between capillary and venous blood. The dilution response curve of the samples was parallel to that of the standard concentration response curve, albeit at the higher end of the acceptable range. However, given that no samples in our dataset had to be diluted relative to other samples (meaning that all DBS samples that are run using this assay are likely to be diluted identically), this is unlikely to be an issue. In addition, the good results of the spike and recovery experiment together with the initial spiking experimental results suggest that the DBS matrix does not contain intrinsic factors that interfere with accurate TRACP-5b measurement. Intra- and inter-plate variability were within the acceptable range for ELISA assays (≤ 10% and ≤ 15-20%, respectively; https://www.salimetrics.com/calculating-inter-and-intra-assay-coefficients-of-variability). The inter-plate variability of 12.1% was on the higher end of the acceptable range, although what is considered acceptable is highly contingent on the intended application of the assay (e.g., clinical diagnoses vs. population-level research; see Semenova et al., 2012). Note that for DBS-based ELISA assays, the inter-plate variability reflects not only the inevitable variability between plates run on separate days because of small variations in temperatures, instrumentation, pipetting, etc., but also the variability in analyte

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measures taken from separate punches of a DBS (George & Moat, 2016), which is not a source of variability when using liquid samples (e.g., serum, supernatant, urine) in ELISAs. The higher limit-of-detection that we measured for this assay than that reported for this assay by its manufacturer highlights the importance of establishing lab-specific LODs when running ELISA assays. Finally, recovery from samples stored at -28°C and controlled room temperature for periods ranging from 1 – 28 days was acceptable, but the overinflated recovery of TRACP-5b after storage at 37°C suggests that at this temperature, TRACP-5b in DBS breaks down into degradation products that are immunoreactive with the capture antibody on the ELISA plates. We therefore recommend storing DBS at -28°C or lower after collection and drying if TRACP-5b is to be measured. TRACP has been observed to lose 20% activity per hour at room temperature unless stabilized (Szulc & Bauer, 2013). Our finding that TRACP-5b levels did not change drastically when stored at room temperature suggests either that the filter paper matrix protected against degradation, as has been demonstrated for some analytes susceptible to degradation by enzymes like hydrolases, esterases, and photocatalytic processes (Freeman et al., 2018), or that the epitopes targeted by the particular coating and detection antibodies used in our ELISA assay were not affected by any potential degradation that may have occurred.

Limitations of our study include using only the MyBiosource TRACP-5b ELISA kit to measure TRACP-5b levels in matched plasma samples rather than additional TRACP-5b ELISA kits from different manufacturers with presumably different antibodies targeting TRACP-5b to verify our findings. In addition, we did not explicitly evaluate the specificity of this assay for TRACP-5b versus TRACP-5a. The manufacturer, however, assured lack of cross-reactivity against TRACP-5a based on proprietary data.

Overall, we have demonstrated the feasibility of measuring TRACP-5b from DBS samples, adding to the arsenal of minimally invasive techniques available to human biologists. Measurement of TRACP5b levels in DBS will open the door to investigating population differences in bone turnover and the effects of factors such as senescence, nutrition, disease, exercise, pregnancy, and lactation on bone mass among non-industrialized populations at low risk for osteoporosis, which can provide important insights into the dynamics of bone formation and bone loss. In addition, this biomarker can be used to gain insight into the relationship between parity and bone density by applying it to natural fertility, subsistence populations for which bone density and high resolution environmental/lifestyle data are available.

AUTHOR CONTRIBUTIONS

GNE and JJS designed the study, directed its implementation, and drafted the manuscript. GNE collected the data and performed analyses. MJD, TJC, PK, and LSS provided logistical support. GNE, MJD, TJC, PK, LSS, and JJS edited the manuscript for content and writing.

ORCID

Geeta N. Eick. http://orcid.org/0000-0001-7512-3265

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FIGURE LEGENDS

Figure 1. Passing-Bablok curves showing the relationship between A) fingerprick DBS (fDBS) and plasma levels of TRACP-5b, B) venous DBS (vDBS) and plasma levels of TRACP-5b, and C) vDBS and fDBS levels of TRACP-5b as measured by ELISA. Passing-Bablok regression equations, the number of samples analyzed, and the male: female (M:F) ratio are indicated in the right hand corners of the graphs.

Figure 2. Bland-Altman plots of the difference in A) plasma and plasma-equivalent fingerprick DBS (fDBS) TRACP-5b values vs. the average of these values (n = 49) and B) fDBS and venous DBS (vDBS) TRACP-5b values vs. the average of these values (n=49). Bias was negligible for both plasma vs. fDBS (0.06) and fDBS vs. vDBS comparisons (0.35), with only one and three points outside the 95 percent confidence intervals indicated by the dotted lines, respectively. These results indicate no significant bias in TRACP-5b measured in plasma vs. TRACP-5b measured in fDBS or TRACP-5b measured in fDBS vs. TRACP-5b measured in vDBS.

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A

Plasma TRACP-5b [IU/L]

fDBS TRACP-5b [IU/L]

y = 1.3x - 0.76
n=37, M:F 14:23

B

Plasma TRACP-5b [IU/L]

fDBS TRACP-5b [IU/L]

y = 1.1x - 0.77
n=36, M:F 14:22

C

fDBS TRACP-5b [IU/L]

fDBS TRACP-5b [IU/L]

y = 0.9x - 0.12
n=44, M:F 19:25

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A Difference plasma - fDBS

Average plasma, fDBS

B Difference vDBS - fDBS

Average vDBS, fDBS

Plasma TRACP-5b [IU/L]

A

fDBS TRACP-5b [IU/L]

y = 1.3x - 0.76
n=37, M:F 14:23

Plasma TRACP-5b [IU/L]

B

vDBS TRACP-5b [IU/L]

y = 1.1x - 0.77
n=36, M:F 14:22

fDBS TRACP-5b [IU/L]

C

vDBS TRACP-5b [IU/L]

y = 0.9x - 0.12
n=44, M:F 19:25