HRS Documentation Report

Documentation of Biomarkers in the 2010 and 2012 Health and Retirement Study

Report prepared by

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Introduction

This document describes the HRS blood-based biomarker data collected and assayed from 2010 and 2012. More detailed descriptions of the procedures for collection of the data and assay of the blood-based markers as well as the genetic markers are available on the HRS website.

Documentation for the 2006 and 2008 data are provided in "**Documentation of Biomarkers in the 2006 and 2008 Health and Retirement Study**." 2013. Eileen Crimmins, Jessica Faul, Jung Ki Kim, Heidi Guyer, Kenneth Langa, Mary Beth Ofstedal, Amanda Sonnega, Robert Wallace and David Weir. **(http://hrsonline.isr.umich.edu/modules/meta/bio2008/desc/Biomarker2006and2008.pdf)**

The description of the samples and the collection procedures for 2006 are provided in a User Guide. **Documentation of Physical Measures, Anthropometrics and Blood Pressure in the Health and Retirement Study**. Crimmins, Eileen M., Heidi Guyer, Kenneth M. Langa, Mary Beth Ofstedal, Robert B. Wallace, and David R. Weir. (2008) (**http://hrsonline.isr.umich.edu/sitedocs/userg/dr-011.pdf**)

Additional useful information on the DBS assays used in HRS is available in "Validation of Blood-based Assays using Dried Blood Spots for use in Large Population Studies." 2014. Eileen Crimmins, Jung Ki Kim, Heather McCreath, Jessica Faul, David Weir, Teresa Seeman, Biodemography and Social Biology, 60: 38-48. **PMCID: PMC4117354**

Blood-based Biomarkers

HRS collected blood-based biomarkers on half the sample in 2006, the other half of the sample provided biomarker data in 2008. The first group was asked for blood samples again in 2010 and the second group gave repeat samples in 2012. The 2010 and 2012 samples each also include a random half of the new cohort enrolled in 2010.

For all years dried blood spot (DBS) samples have been assayed for 5 biomarkers:

a. Total and HDL cholesterol, indicators of lipid levels
b. Glycosylated hemoglobin (HbA1c) – an indicator of glycemic control over the past 2-3 months
c. C-reactive protein (CRP), a general marker of systemic inflammation
d. Cystatin C, an indicator of kidney functioning.

Sample and Methods of Collection

Sample

The blood tests were intended for all of those who were available for the Enhanced Face-to-Face Interview (EFTF) interview. Special informed consent was acquired for the blood acquisition process.

Consent Rate

The blood spot consent rate in 2010 was 85%. The completion rate, conditional on consent, was 99%. The overall completion rate was 84%. In 2012, the blood spot consent rate in was 87%. The completion rate, conditional on consent, was 99% resulting in an overall completion rate of 86%.

Shipping

In 2010 and 2012, interviewers mailed the DBS cards directly to the University of Michigan, where they were sorted, frozen and shipped (in batches) to the appropriate lab for processing.

Laboratories Used and Assays Conducted

A series of labs have been used over the years to assay HRS DBS.
In 2006

- **a.** Biosafe Laboratories – assayed HbA1C, Total cholesterol and HDL cholesterol
- **b.** The University of Vermont assayed CRP and Cystatin C

In 2008

- **a.** Two labs performed HbA1c assays - Biosafe and FlexSite (after the bankruptcy of Biosafe)
- **b.** Two labs assayed Total and HDL Cholesterol - Biosafe and the University of Washington (after the bankruptcy of Biosafe)
- **c.** The University of Vermont assayed CRP and Cystatin C

In 2010

- **a.** Heritage Laboratory assayed Total and HDL cholesterol and HbA1c
- **b.** The University of Washington assayed CRP and cystatin C

In 2012

- **a.** The University of Washington performed all 5 assays

Heritage Laboratories (now owned by Clinical Reference Laboratory, Inc.)
1111 West Old 56 Hwy.
Olathe, KS 66061
CLIA Registration No. 17D0943396
College of American Pathologist Registration No. 6909601

University of Washington Department of Medicine Dried Blood Spot Laboratory
Immunology Division, Department of Laboratory Medicine
Director: Mark H. Wener, MD, wener@u.washington.edu
Project Director Alan Potter, Ph.D., apotter@uw.edu

Description of HRS Blood-Based Biomarker Data

Dried Blood Spots vs. Whole Blood

Because the resulting biomarker values based on DBS vary across assays and laboratories and may be quite different from the more conventionally used whole blood assays such as those in NHANES, and because many analysts want to make comparisons to such standard assays, we compare our results to those from NHANES. We have also constructed and released a variable for each assay, which we call an NHANES equivalent value. **We recommend the NHANES equivalent assay values for analytic use.** These variables were constructed by assuming that the distribution of the DBS assays is similar to that in NHANES; we determine the value of both assays at each percentile; and then transform the DBS assays into the NHANES scale after adjusting for any between-lab differences.
Comparison of the HRS DBS values and those from venous blood assays is described in detail in ***Results from the Health and Retirement Study Biomarker Validation Project*** (Crimmins et al. 2013, DRAFT), *Validation of Blood-based Assays using Dried Blood Spots for use in Large Population Studies* (Crimmins et al. 2014) and the HRS Documentation for the 2006 and 2008 blood-based assays. These sources make it clear that different lab assays and procedures result in different assay values. As mentioned above and described more below, the HRS solution to the problem of different assays is to produce an NHANES Equivalent Value.

Constructing NHANES Equivalent Values

The NHANES equivalent values make the assay levels for the HRS data based on DBS similar to the level in NHANES where values are based on conventional assays while the variability in the HRS sample is preserved. Because the weighted NHANES and HRS samples are both population-based studies intended to represent the non-institutional U.S. population, we adjust the HRS DBS values to levels consistent with NHANES, exploiting the fact that the population distributions should be the same if there are no differences in lab procedures. We did this for the HRS 2006 and 2008 assays using a pooled sample for NHANES 2005-2006 and NHANES 2007-2008. For HRS 2010 and 2012, we pool the NHANES samples for NHANES 2009-2010 and 2011-2012. (For CRP we use only 2009-2010 as 2011-2012 is not yet available.) **This means that average differences in HRS samples from 2006 to 2010 and from 2008 to 2012 will reflect differences, or change over time, in NHANES. Because cystatin C has not been regularly done in NHANES, we use the same comparison sample for cystatin C in 2010 and 2012 as for the 2006 and 2008 values, so there is no time change in this measure.**

Our approach is to first calculate the values of the assays corresponding to (weighted) 100 percentiles in HRS and in NHANES. For HRS we use the biomarker weights (MBIOWGTR and NBIOWGTR). [To facilitate construction of percentiles when values are discrete and have many individuals scored at the same value, we first add a very small random number to each observed value, create the (weighted) percentiles based on the altered values, and then take the mean of the actual assay values at each percentile]. For NHANES, we pool the 2009-10 and 2011-12 samples for assays other than CRP and cystatin C. For cystatin C, the NHANES comparison data are from 1999-2002 and are the same data used for the HRS 2006 and 2008 assays. We then have 100 percentiles for HRS and 100 percentiles for NHANES. (Because of the highly skewed distribution of CRP, we work with log values). We then

regress the HRS value on the NHANES value to create an equation that can be used to convert HRS values into NHANES Equivalent values.

Descriptive Data on HRS Biomarkers

This section begins with an overview that presents descriptive statistics on each of the five biomarkers from the HRS 2010 and 2012 data for the 50+ HRS sample with nonzero weights. We also provide some comparison with NHANES values (for the 50+ NHANES sample) which are based on conventional venous blood assays. We also provide descriptive statistics for the HRS NHANES equivalent values. In subsequent sections, we provide details on each assay. The following tables provide means, standard deviations and the minimum and maximum values for each of the five biomarkers in HRS 2010 and 2012 followed by NHANES 2009-2012. The HRS data are weighted using the wave appropriate biomarker weight.

HRS 2010	N	Mean	SD	Min	Max
HbA1C (%) MA1CHER	7641	6.04	1.06	3.90	18.10
Total Cholesterol (mg/dL) MTCHER	7541	197.03	33.20	117.00	337.00
HDL (mg/dL) MHDLHER	7540	56.29	14.68	19.00	119.00
Cystatin C (mg/L) MCYSCUW	7544	0.75	0.34	0.04	6.24
CRP (ug/mL) MCRPUW	7528	3.48	7.27	0.06	177.41
Logged CRP	7528	0.51	1.18	-2.83	5.18
NHANES 2009-12					
HbA1C (%)	4405	5.81	1.03	4.00	17.80
Total Cholesterol (mg/dL)	4604	195.10	45.50	75.00	528.00
HDL (mg/dL)	4604	53.86	16.83	11.00	175.00
Cystatin C (1999-2002) (mg/L)	3285	1.08	0.50	0.43	10.70
CRP (2009-2010) (ug/mL)	2388	3.53	6.56	0.10	113.20
Logged CRP (2009-2010)	2388	0.23	1.48	-2.30	4.73
HRS 2010 NHANES Equivalent Value[1]					
HbA1C (%) MA1C_ADJ	7641	5.82	1.01	3.78	17.26
Total Cholesterol (mg/dL) MTC_ADJ	7541	195.20	43.09	91.32	376.87
HDL (mg/dL) MHDL_ADJ	7540	53.82	15.82	13.64	121.40
Cystatin C (mg/L) MCYSC_ADJ	7544	1.08	0.49	0.05	9.09
CRP (ug/mL) MCRP_ADJ	7528	3.63	7.59	0.05	185.36
Logged CRP	7528	0.54	1.19	-2.95	5.22

[1]1999-2002 for Cystatin C; 2009-2010 for CRP

HRS 2012	N	Mean	SD	Min	Max
HbA1C (%) NA1CUW	6993	5.52	0.64	3.70	12.30
Total Cholesterol (mg/dL) NTCUW	7206	299.18	67.76	129.00	990.00
HDL (mg/dL) NHDLUW	7151	84.16	22.15	41.00	213.00
Cystatin C (mg/L) NCYSCUW	6720	0.61	0.29	0.05	5.80
CRP (ug/mL) NCRPUW	6976	4.27	8.83	0.06	244.67
Logged CRP	6976	0.72	1.17	-2.81	5.50
NHANES 2009-12[1]					
HbA1C (%)	4405	5.81	1.03	4.00	17.80
Total Cholesterol (mg/dL)	4604	195.10	45.50	75.00	528.00
HDL (mg/dL)	4604	53.86	16.83	11.00	175.00
Cystatin C (1999-2002) (mg/L)	3285	1.08	0.50	0.43	10.70
CRP (2009-2010) (ug/mL)	2388	3.53	6.56	0.10	113.20
Logged CRP (2009-10)	2388	0.23	1.48	-2.30	4.73
HRS 2012 NHANES Equivalent value[1]					
HbA1C (%) NA1C_ADJ	6993	5.78	0.98	3.01	16.14
Total Cholesterol (mg/dL) NTC_ADJ	7206	195.23	43.79	85.24	641.68
HDL (mg/dL) NHDL_ADJ	7151	53.84	15.81	23.03	145.81
Cystatin C (mg/L) NCYSC_ADJ	6720	1.13	0.49	0.20	9.76
CRP (ug/mL) NCRP_ADJ	6976	3.60	7.30	0.13	202.40
Logged CRP	6976	0.61	1.09	-2.07	5.31

[1] 2009-10 for CRP, 1999-2002 for Cystatin C

Sample Weights

Separate sample weights exist for the biomarker sub-sample in each wave. These weights, MBIOWGTR for 2010 and NBIOWGTR for 2012, can be found in the Cross-Wave Tracker File and in the biomarker data file for each collection wave.

The biomarker sample weight is the product of the HRS core sampling weight and a non-response adjustment factor. The HRS sampling weight from the concurrent interview was used as the base weight. The nonresponse adjustment factor was obtained from a propensity model predicting the probability of completing the biomarker portion of the eFTF interview among those selected and eligible to participate. The propensity model was estimated by logistic regression and weighted by the

base weight. Predictor variables included age, sex, race/ethnicity, education, coupleness, self-rated health, number of physical limitations and report of a chronic health condition (i.e., diabetes, use of diabetes medications, hypertension, heart conditions, myocardial infarction, angina, congestive heart failure or stroke). Predictor variables were taken from the current interview. The inverse of the fitted probability of completion formed the non-response adjustment factor. Finally, the weights were post-stratified to closely match the HRS sample composition by age, gender, and race.

Appendix A – Laboratory Technical Reports

Description of Assays: This material is derived from technical reports provided by the assay suppliers.

Heritage Laboratory

Appraise DBS Hemoglobin A1C test

The Appraise® Hemoglobin A1c test is based on a dried blood spot technique and the performance of the assay has been compared with the reference Primus Affinity chromatography method using paired whole blood specimens.

Test Performance Specifications
Precision: Within-assay variability was determined by testing dried blood spots containing four concentrations of hemoglobin A1c. All specimens were tested twenty times. The error rate at each Hemoglobin A1c level is presented below:

Mean Hemoglobin A1c (%)	Standard Deviation	Coefficient of Variation (%)
5.6	0.1	1.8
7.3	0.0	0.0
8.7	0.0	0.0
10.4	0.0	0.0

Within-day error (repeatability) as well as between-day error (reproducibility) are also within acceptable limits (data not shown).

Linearity and Range: The linearity of Hemoglobin A1c was analyzed over a measured range of 3.10-16.23%. The maximum deviation from linearity was 1.3%.
Expected Values: A population of apparently healthy persons was analyzed using the Appraise® Hemoglobin A1c methodology. The expected values are less than 7.0%.

Specimen Stability: Appraise® Hemoglobin A1c specimens are stable for not less than 30 days when stored at room temperature and longer when stored refrigerated at 2-8°C.

Accuracy: Paired blood collected in lavender top tubes versus Appraise® collected blood samples containing varying concentrations of Hemoglobin A1c were analyzed to determine comparability of the two collection methods using linear regression. The R2 of the regression = 0.9979.

Specimen Requirements: The Appraise® Hemoglobin A1c test system requires a few drops of blood to saturate the surface area of a specialized paper card. The blood is dried and placed into a waterproof carrier envelope for mailing to Heritage Labs.

University of Washington

Cholesterol Assays

The DBS assay for **total cholesterol (TC)** done at the University of Washington is a fluorimetric assay. A punch from a DBS card is eluted with a buffer solution. The elution solution is incubated with assay reagent containing cholesterol ester hydrolase, cholesterol oxidase, peroxidase, and a fluorogen. The cholesterol ester hydrolase catalyzes the conversion of cholesterol esters to cholesterol, and this and de novo cholesterol is oxidized by cholesterol oxidase, producing hydrogen peroxide (H_2O_2) as a byproduct. In the presence of the peroxidase, the H_2O_2 reacts with the non-fluorescent fluorogen to produce a fluorescent fluorophore. The TC concentration is directly proportional to the fluorescent intensity of the solution; fluorescence is measured spectrophotometrically. A standard curve is constructed by plotting the fluorescence values of the standards against the known TC concentrations. Using the standard curve, the fluorescence values of the QC samples and patient samples are read as TC concentrations. Acceptability of the assay is determined by comparing the TC concentrations of the QC samples with their established values.

DBS TC assay calibrators were constructed from high TC concentration pooled human plasma (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) serially diluted with 7% bovine serum albumin in phosphate buffered saline (BSA/PBS; Sigma Aldrich, St. Louis, MO) to the desired final TC concentration. Two DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high TC concentration QC sample) or diluted with BSA/PBS (low TC concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at room temperature (RT) (23°c). The final TC concentration of each calibrator and QC sample solution was determined by analysis on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well (Greiner Bio-One, Monroe, North Carolina). The samples were either immediately assayed or the microtiter plates were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. A TC elution buffer comprised of 400µl ddH_2O with 0.1% Triton X-100 (Sigma) was added to each microtiter plate well. The plate was sealed and gently shaken for 1hr on a microplate shaker (Delfia Plateshake, PerkinElmer, Waltham, MA) to elute TC from each punch. 20 µl of eluent was transferred from each well of the elution plate to an assay microtiter plate. This was followed by addition of 100 µl of TC assay reagent comprised of 70% Cholesterol Chromogen (Synermed, Westfield, IN), 29% Cholesterol Enzyme (Synermed) and 1% Cayman ADHP (10-acetyl-3,7-dihydroxyphenoxazine; Cayman Chemical, Ann Arbor, MI). The assay plate was gently shaken for 30sec and then incubated at 37°c for 30min. The fluorescence intensity (RFU) of each well was read at 530/25nm excitation and 590/35nm emission on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). A linear regression calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded fluorescence values, was used to convert the blank-subtracted RFU value of each sample into a DBS TC concentration (Gen5 Software, BioTek).

The TC assay lower limit of detection is 27mg/dl, within-assay imprecision (CV) is 4.0% and between-assay imprecision is 4.7%. The TC concentrations of 105 DBS samples analyzed by the DBS assay correlated with the TC concentrations of paired plasma samples (Pearson R = 0.93) and were linearly related (DBS assay TC value = 17.141 + plasma-equivalent TC value X 1.582). Correction for hematocrit did not improve the agreement between the serum and DBS TC values.

The DBS assay for **HDL cholesterol (HDL)** done at the University of Washington is a fluormetric assay. A punch from a DBS card is eluted with deionized water. The elution solution is combined with a reagent containing anti-human β-lipoprotein antibody (to bind non-HDL lipoproteins into nonreactive complexes) and a flurogen. A second reagent, containing cholesterol ester hydrolase, cholesterol oxidase and peroxidase, is then added. The cholesterol ester hydrolase catalyzes the conversion of HDL-C into cholesterol, which is in turn oxidized by cholesterol oxidase and produces hydrogen peroxide (H_2O_2) as a byproduct. In the presence of the peroxidase, H_2O_2 reacts with the non-fluorescent fluorogen to produce a fluorescent fluorophore. The HDL-C concentration is directly proportional to the fluorescent intensity of the solution; fluorescence is measured spectrophotometrically. A standard curve is constructed by plotting the fluorescence values of the standards against the known HDL-C concentrations. Using the standard curve, the fluorescence values of the QC samples and respondent samples are read as HDL-C concentrations. Acceptability of the assay is determined by comparing the HDL-C concentrations of the QC samples with their established values.

DBS HDL-C assay calibrators were constructed from high HDL-C concentration pooled human plasma (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) serially diluted with 7% bovine serum albumin in phosphate buffered saline (BSA/PBS; Sigma Aldrich, St. Louis, MO) to the desired final HDL-C concentration. Two DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high HDL-C concentration QC sample) or diluted with BSA/PBS (low HDL-C concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final HDL-C concentration of each calibrator and QC sample solution was determined by analysis on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter plate well (Greiner Bio-One, Monroe, North Carolina). The samples were either immediately assayed or the microtiter plates were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 400µl diH_2O HDL-C elution buffer was added to each microtiter plate well. The plate was sealed and gently shaken for 1hr on a microplate shaker (Delfia Plateshake, PerkinElmer, Waltham, MA) to elute HDL-C from each punch. 40 µl of eluent was transferred from each well of the elution plate to an assay microtiter plate. This was followed by addition of 75 µl of HDL-C assay reagent 1 comprised of 99% EZ HDL Cholesterol Reagent 1 (Trinity Biotech, St Louis, MO) and 1% Cayman ADHP (10-acetyl-3,7-dihydroxyphenoxazine; Cayman Chemical, Ann Arbor, MI). The assay plate was gently shaken for 30sec and then incubated at 37°c for 25min. At the conclusion of this incubation, 25 µl of HDL-C assay reagent 2 (EZ HDL Cholesterol Reagent 2; Trinity Biotech) was added, the assay plate shaken for 30sec and then incubated at 37°c for 30min. The fluorescence intensity (RFU) of each well was

read at 530/25nm excitation and 590/35nm emission on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). A linear regression calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded fluorescence values, was used to convert the blank-subtracted RFU value of each sample into a DBS HDL-C concentration (Gen5 Software, BioTek).

The HDL-C assay lower limit of detection is 15mg/dl, within-assay imprecision (CV) is 5.3% and between-assay imprecision is 9.9%. The HDL-C concentrations of 104 DBS samples analyzed by the DBS assay correlated with the HDL-C concentrations of paired plasma samples (Pearson R = 0.88) and were linearly related (DBS assay HDL-C value = 32.941 + plasma-equivalent HDL-C value X 0.965). Correction for hematocrit did not improve the agreement between the serum and DBS HDL-C values.

Hemoglobin A1c

The DBS **hemoglobin A1c (HbA1c) Assay** uses an automated ion-exchange high-performance liquid chromatography (IE-HPLC) system to measure the percentage of glycosylated hemoglobin HbA1c in dried blood spot (DBS) samples. A 3.2mm diameter disc punched from a DBS card containing a patient blood sample is eluted in a buffer solution. The elution solution is transferred to a sample vial, diluted and analyzed on a Bio-Rad Variant II Hemoglobin Testing System. The percentage of glycosylated HbA1c relative to total HbA is determined by chromatographic separation of glycosylated HbA1c on a cation exchange cartridge. Bio-Rad proprietary software is used to generate a standard curve from vendor-provided calibrators to establish that the instrument is identifying the known glycosylated HbA1c values in vendor-provided liquid quality control (QC) samples as well as in laboratory-created DBS QC samples. The percentage of glycosylated HbA1c in a patient sample is automatically determined by peak curve integration. Acceptability of the assay is determined by the comparison of the glycosylated HbA1c values of the QC samples with their established values.

DBS samples are stored upon receipt at the lab at -80°c (Thermo Scientific Revco Ultima Plus, Fisher Scientific, Pittsburg, PA). Immediately prior to processing, DBS are warmed to RT and then a single 3.2mm (1/8in) diameter disc is punched from each DBS using a BSD700 Semi-Automated Dried Sample Puncher (BSD Robotics, Brisbane, QLD, Australia) into a deep-96 well microtiter plate well (Greiner Bio-One, Monroe, NC). Microtiter plates are then either immediately assayed or are firmly sealed (CapMat, Greiner Bio-One) and stored at -80°c pending assaying.

Immediately prior to assaying, microtiter plates are warmed to RT. 100µl HbA1c elution buffer (Bio-Rad Hemoglobin A1c Program Wash/Diluent Reagent, Bio-Rad Laboratories, Hercules, CA) is added to each microtiter plate well. The plate is sealed and vigoroulsy shaken for 1hr on a Delfia Plateshake microplate shaker (PerkinElmer, Waltham, MA). The eluent is transferred from each well of the elution plate to a Bio-Rad HbA1c sample vial (Bio-Rad Laboratories) containing 500 µl of Wash/Diluent Reagent, gently shaken for 30sec and then placed on a Bio-Rad Variant II Hemoglobin Testing System (Bio-Rad Laboratories). The Variant II performs a chromatographic separation of glycosylated HbA1c on a cation exchange cartridge by applying a programmed buffer gradient of increasing ionic strength to separate hemoglobins based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of a filter photometer where absorbance at 415nm is measured (background absorbance corrected at 690nm). Bio-Rad Variant II

proprietary software (Variant II Clinical Data Management Software, Bio-Rad Laboratories) is used to calculate the slope and intercept of the standard curve generated by analysis of the Bio-Rad calibrators. This data is then used by the software to determine the %HbA1c of each sample based on the glycosylated HbA1c characteristic retention time and the glycosylated HbA1c peak area. Acceptability of the assay is determined by comparing the %HbA1c concentrations of two QC samples (Lyphochek Bilevel Diabetes Control, Bio-Rad Laboratories) and laboratory created DBS QC samples at the beginning, middle, and end of each assay run against established values. An assay is rejected if the mean value of a control sample is greater than 3SD above or below the established value, or if the mean value of one control sample is greater than 2SD above the established value and the mean value of a second control sample is greater than 2SD below the established value. Acceptability of the analysis of each sample is determined by examining the chromatogram for proper form, absence of interfering peaks, acceptable total area, and %HbA1c value within the analytical measurement range.

The HbA1c assay analytical measurement range is 3.1% to 18.5% per limits established by Bio-Rad Laboratories. The within-assay imprecision (CV) is 1.4% and between-assay imprecision is 2.2%. The %HbA1c values of 189 DBS samples analyzed in duplicate by the DBS assay correlated with the %HbA1c values of DBS-matched liquid blood samples measured in duplicate (Pearson R = 0.995) and were linearly related (blood %HbA1c value = -0.918 + DBS %HbA1c value X 1.192).

Cystatin C Assay

The DBS **cystatin C (cysC)** assay is a sandwich ELISA used to measure the cysC concentration in dried blood spot (DBS) samples. A filter paper disc is punched from a DBS card containing a cysC assay calibrator, a quality control (QC) sample or a study sample is eluted in a buffer solution. The elution solution from each sample is transferred to a well on an ELISA microtiter plate. The bottom face of each well of the plate is pre-coated with a polyclonal antibody (pAb) that recognizes distinct antigenic determinants on the cysC molecule. cysC in the elution solution is bound by the anti-cysC pAb (solid phase immobilization). A conjugate solution containing anti-cysC pAb coupled to peroxidase (enzyme-linked antibody) is then added to each well, resulting in cysC molecules being sandwiched between the solid phase and enzyme-linked antibodies. After incubation, the wells are washed to remove unbound material. A tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution is added; H_2O_2, cleaved by the peroxidase, reacts with TMB and causes the solution to develop color**.** The reaction is stopped by adding an acid reagent after a fixed period of time. The cysC concentration is directly proportional to the absorbance of the solution; absorbance is measured spectrophotometrically. A standard curve is constructed by plotting the absorbance values of the calibrators against their known cysC concentrations. Using the standard curve, the absorbance values of the QC samples and patient samples are read as cysC concentrations. Acceptability of the assay is determined by comparing the cysC concentrations of the QC samples with their established values.

DBS cysC assay calibrators were constructed from whole blood obtained from a single donor (Puget Sound Blood Center (PSBC), Seattle, WA). High cysC concentration plasma was created by pooling high cysC concentration plasma (University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) and spiking with recombinant human cysC concentrate (Sigma-Aldrich; St. Louis, MO). cysC calibrators were created by centrifuging aliquots of the whole blood and replacing volumes of plasma with equal volumes of high cysC concentration plasma; increasing

volumes of plasma were replaced to create increasing cysC concentration calibrators. Low cysC concentration calibrators were created by replacing volumes of plasma with equal volumes of 5% Human Albumin Solution (ZLB Behring; Berne, Switzerland); increasing volumes of plasma were replaced to create decreasing cysC concentration calibrators. DBS QC samples were constructed from whole blood (PSBC) obtained from a donor with a high cysC concentration (high cysC QC sample) and from whole blood obtained from a donor with a low cysC concentration (low cysC QC sample). 75µl aliquots of each calibrator and QC sample whole blood was pipetted onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final cysC concentration of each calibrator and QC sample solution was determined from analyses of plasma taken from the calibrator and QC sample whole blood on a Seimens Dade-Behring BN II Nephelometer (Deerfield, IL). DBS calibrators, QC samples and study samples, sealed in Ziploc bags with desiccant packs were stored upon receipt at -80°c (Thermo Scientific Revco Ultima Plus, Fisher Scientific, Pittsburg, PA) until processing. Each study sample consisted of a DBS card containing a series of 50µl drops of whole blood obtained from a finger-stick placed singly on five separate areas of a Protein Saver Card No. 903 filter paper (Whatman) and dried at RT. Immediately prior to processing, DBS calibrators, QC samples and study samples were warmed to RT and then a single 3.2mm (1/8in) diameter punch was punched from each DBS using a BSD700 Semi-Automated Dried Sample Puncher (BSD Robotics, Brisbane, QLD, Australia) into a deep-well microtiter plate well (Greiner Bio-One, Monroe, NC). Microtiter plates were either immediately assayed or were firmly sealed (CapMat, Greiner Bio-One) and stored at -80°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 400µl cysC elution buffer (cysC Dilution Buffer; BioVendor, Candler, NC) was added to each microtiter plate well. The plate was sealed and vigorously shaken for 1hr at RT on a Delfia Plateshake microplate shaker (PerkinElmer, Waltham, MA), held overnight at 4°c, and then vigorously shaken for 1hr at RT to elute cysC. 100 µl of eluent was transferred from each well of the elution plate to an ELISA microtiter plate (RD191009100R; BioVendor), the plate was gently shaken for 60min at RT in the dark and then washed 5X with cysC Wash Solution (BioVendor). 100 µl of Enzyme Conjugate Solution (BioVendor) was added, the plate gently shaken for 30min at RT in the dark and then washed 5X with cysC Wash Solution (BioVendor). 100 µl of TMB Reagent (BioVendor) was added and the plate was gently shaken for 12min at RT in the dark. 100 µl of Stop Solution (BioVendor) was added and the plate was gently shaken for 30min at RT in the dark. The plate was then placed on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). The absorbance of each well (OD) was read at 450nm excitation. A 5-parameter weighted calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded absorbance values, was used to convert the OD value of each sample into a DBS cysC concentration (Gen 5 Software, BioTek). Acceptability of the assay was determined by comparing the cysC concentrations of the QC samples with their established values. An assay would be rejected if the mean value of a control sample was greater than 3SD above or below the established mean value, or if the mean value of two control samples was each greater than 2SD above or below the respective established mean value.

The cysC assay lower limit of detection is 0.025 µg/ml, within-assay imprecision (CV) is 2.6% and between-assay imprecision is 5.6%. The cysC concentrations of 91 DBS samples analyzed in duplicate by the DBS assay correlated with the cysC concentrations of DBS-matched serum samples measured in duplicate by nephelometry (Pearson R = 0.961) and were linearly related (serum cysC value = 0.256 + DBS cysC value X 0.999).

High-Sensitivity C-Reactive Protein (hsCRP) Assay

The DBS high-sensitivity **C-Reactive Protein (hsCRP)** assay is a sandwich ELISA. A punch from a dried blood spot (DBS) card containing either a CRP assay standard, a quality control (QC) sample or a patient sample is eluted in a buffer solution. The elution solution is transferred to an ELISA microtiter plate. The bottom face of each well of the ELISA plate is pre-coated with an anti-CRP monoclonal antibody (mAb) that binds CRP in the elution solution (solid phase immobilization). A conjugate solution containing anti-CRP Ab coupled to peroxidase (enzyme-linked antibody) is then added to each well resulting in CRP molecules being sandwiched between the solid phase and enzyme-linked antibodies. A tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) solution is added; H_2O_2, cleaved by the peroxidase, reacts with TMB and causes the solution to develop a blue color**.** The CRP concentration is directly proportional to the absorbance of the solution; absorbance is measured spectrophotometrically. A standard curve is constructed by plotting the absorbance values of the standards against the known CRP concentrations. Using the standard curve, the absorbance values of the QC samples and patient samples are read as CRP concentrations. Acceptability of the assay is determined by comparing the CRP concentrations of the QC samples with their established values.

DBS CRP assay calibrators were constructed from pooled human plasma with a negligible CRP concentration (negligible CRP plasma; University of Washington Department of Laboratory Medicine, Seattle, WA; UW Lab Med) spiked with CRP concentrate (Cell Sciences, Canton, MA) and serially diluted with negligible CRP plasma to the desired final CRP concentrations. Three DBS QC samples were constructed from a separate pool of human plasma, either undiluted (high CRP concentration QC sample) or diluted with negligible CRP plasma (medium CRP concentration QC sample and low CRP concentration QC sample). Each calibrator and QC sample solution was mixed with a constant volume of washed human erythrocytes (UW Lab Med), pipetted in 75µl aliquots onto No. 903 filter paper (Whatman, Piscataway, NJ) and dried for 4hr at RT (23°c). The final CRP concentration of each calibrator and QC sample solution was determined by analysis on a UniCel DxC 800 Synchron Clinical System (Beckman Coulter, Miami, FL). DBS calibrators, QC samples and study samples were sealed in Ziploc bags with desiccant packs and stored at -70°c until processing. Immediately prior to processing, DBS were warmed to RT and a single 3.2mm (1/8in) diameter punch was punched from each DBS into a microtiter elution plate well (Greiner Bio-One, Monroe, North Carolina). The samples were either immediately assayed or the microtiter plates were firmly sealed and stored at -70°c pending assaying.

Immediately prior to assaying, microtiter plates were warmed to RT. 200µl CRP elution buffer (hsCRP Sample Diluent; Percipio, Inc, Manhattan Beach, CA) was added to each plate well. The plate was sealed and gently shaken for 1hr on a Delfia Plateshake microplate shaker (PerkinElmer, Waltham, MA) to elute CRP. 20 µl of eluent was transferred from each well of the elution plate to an ELISA microtiter plate followed by addition of 100 µl of CRP Enzyme Conjugate Reagent (Percipio). The ELISA plate was gently shaken at RT for 45min and then washed 5X with di/ddH_2O. 100 µl of TMB Reagent (Percipio) was added to each well and the plate placed on a microtiter plate reader (Synergy HT, BioTek, Winooski, VT). The absorbance of each well (OD) was read at 370nm excitation after the OD of the high standard on the plate reached 1.5 (approximately 20min). A 5-parameter weighted calibration curve, constructed by plotting the assigned concentrations of the calibrators against the recorded absorbance values, was used to convert the blank-subtracted OD value of each sample into a DBS CRP concentration (Gen 5 Software, BioTek).

The CRP assay lower limit of detection is 0.035mg/L, within-assay imprecision (CV) is 8.1% and between-assay imprecision is 11.0%. The CRP concentrations of 87 DBS samples analyzed by the DBS assay correlated with the CRP concentrations of paired plasma samples (Pearson R = 0.99) and were linearly related (DBS assay CRP value = 0.370 + plasma-equivalent CRP value X 1.077).