C-Reactive Protein

Dried Blood Spot C-reactive protein Measurement in Wave I of the National Social Life Health & Aging Project

Authors:

Bhairavi Nallanathan, University of Chicago, Department of Ob/Gyn

Thomas McDade, Northwestern University, Departments of Anthropology and Cells to Society (C2S)

Sharon Williams, Purdue University, Department of Anthropology

Stacy Tessler Lindau, MD, MAPP, University of Chicago, Departments of Ob/Gyn and Medicine – Geriatrics, Chicago Core on Biomeasures in Population-Based Health and Aging Research at NORC and the University of Chicago Center on Demography and Economics of Aging*

* Corresponding author.
E-mail address: slindau@uchicago.edu (S.T. Lindau).
Fax: +1 773 834 5664

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Rationale

C-reactive protein (CRP) is an acute phase protein that is a central component of the inflammatory response to injury or infection. CRP production is regulated by cytokines such as IL-6 and is primarily synthesized by hepatocytes. Since the half life of plasma CRP (approximately 19 hrs) remains constant under conditions of both health and disease, the only factor that influences the concentration of CRP is the rate of synthesis. This rate is a direct reflection of the intensity of the pathological processes occurring in the body (Pepys & Hirschfield, 2003). CRP is primarily used as a marker of inflammation and has emerged as an important predictor of cardiovascular risk in both men and women (Pearson et al., 2003; Ridker, 2003b; Ridker, Hennekens, Buring, & Rifai, 2000). Minor elevation of CRP is useful in prognosticating many conditions, particularly age-related disease, and is useful in predicting mortality in both diseased and healthy individuals (Kushner, Rzewnicki, & Samols, 2006). The sensitivity provided by conventional assays for CRP is insufficient to detect levels for vascular disease. This led to the development of high-sensitivity CRP assays which are now widely available (Ridker, 2003a). The CDC has recommended the following with regards to classification of cardiovascular risk when interpreting CRP values from serum or plasma: Low risk: CRP < 1.0 mg/L; Normal risk: CRP >= 1.0 mg/L, <= 3.0 mg/L; High risk: CRP > 3.0 mg/L; Outliers: CRP > 10 mg/L should be excluded from most analyses due to the apparent presence of acute, active infection (Pearson et al., 2003).

Some studies report an increase in CRP levels in both men and women with increasing age (Hutchinson et al., 2000; Khor et al., 2004). Other studies have found elevated CRP levels in women in comparison to men (Khera et al., 2005; McConnell et al., 2002). Even after controlling for confounding variables, women still had significantly higher levels of CRP. Increased BMI is associated with higher levels of CRP and is more pronounced in women than in men (Khera et al., 2005). Postmenopausal women on hormone replacement therapy were also found to have higher levels of CRP in comparison to nonusers (Ridker, Hennekens, Rifai, Buring, & Manson, 1999). Many studies have been conducted that link CRP levels to other medical conditions, demographic, and socioeconomic factors (Kushner et al., 2006).

Measurement

In the clinical setting, serum or plasma samples assayed for CRP are usually collected through venipuncture. This method is invasive and not feasible for conducting large population-based studies since the procedure requires trained phlebotomists and samples would need to be immediately processed and stored under controlled conditions. The dried blood spot method offers a convenient and minimally invasive alternative for collecting blood samples that can be used to assay CRP. In NSHAP, whole blood was collected by finger-stick, using a disposable lancet, and then applied to filter paper for transport and storage. (Details about sample collection procedures are described in the “Specimen Collection” section, below.)

There is a strong linear relationship between concentrations of CRP measured in serum and dried blood spot samples. Based on a study validating the CRP method used by NSHAP, Pearson R= 0.98 (n= 84 paired samples) (McDade, Burhop, & Dohnal, 2004). Another study reported a correlation of .99 (n=101) and found no significant difference in mean CRP concentrations in serum, finger puncture, and venipuncture blood spots (F=0.007, p=0.99) (Cordon, Elborn, Hiller, & Shale, 1991).

Population Norms

Table 1. C-reactive protein distribution in NHANES III (Wong, Pio, Valencia, & Thakal, 2001) Age range: 30-74 years*

CRP Percentiles (%)		Male CRP (mg/L) (N=4472)	Female CRP (mg/L) (N=5212)
Median	5	2.10	2.10
	10	2.10	2.10
	25	2.10	2.10
	50	2.10	2.10
	75	3.30	6.0
	90	7.70	1.10
	95	11.0	17.0
Mean ±SD		4.10 ± 6.40	5.50 ± 9.10

Table 2.a Gender differences in median plasma CRP levels (mg/L) (Lakoski et al., 2006) Values were obtained using BNII nephelometer (N High sensitivity CRP; Dade Behring Inc, Deerfield, IL)

	N	Age (SD)	Median Plasma CRP Levels (mg/L)
Men	3193	62.1±10	1.43
Women	2625	62.1±10	2.15

Table 2.b Gender differences in log of mean values (mg/L) of CRP levels in plasma (Taaffe, Harris, Ferrucci, Rowe, & Seeman, 2000). Values were obtained using ELISA (Macy et al, 1997)

	N	Age (SD)†	Log of Mean (mg/L) Plasma CRP Levels
Men	412	74.3 ± 2.7	0.66
Women	468	74.3 ± 2.7	0.58

† Mean age for both men and women

Table 3. CRP levels in men and women with and without history of cardiovascular (CV) events. Values for men (Ridker et al., 1997) was obtained using ELISA (Calbiochem). Values for women (Ridker et al., 2000) was obtained using Latex enhanced immunonephelometric assays on NB II analyzer (Dade Behring, Newark, Del.)

		N	Age (SD)	Median Plasma CRP Levels (mg/L)	Range	P*
No CV Events	Men	543	59 ± 9.1	1.130	-	<0.001‡
	Women	244	59.3	2.80	1.1-5.5	<0.001†
CV Events	Men	543	59 ± 9.2	1.40	-	<0.001‡
	Women	122	59.3	4.20	2.1-8.3	<0.001†

Table 4. Effects of Hormone Replacement Therapy (HRT) on CRP levels in postmenopausal women (Ridker et al., 1999). Values were obtained using high-sensitivity assay with a coefficient of variation below 5% (hs-CRP, Dade Behring)

	N	Age (SD)	Median Plasma CRP Levels (mg/L)	P
HRT	182	52.4 (5.3)	2.70	0.001
No HRT	311	49.6 (4.9)	1.40	

Table 5. NSHAP Summary statistics for C-reactive protein levels (mg/L)

	Range	Median (mg/L) (weighted)	25%-75%
Men			
Ages 57-64	0-38.2	1.47	0.63-3.36
Ages 65-74	0.06-100	1.29	0.52-3.05
ages 75+	0.03-39.8	1.14	0.48-2.54
Women			
Ages 57-64	0-100	1.92	0.70-4.85
Ages 65-74	0-41.8	1.69	0.77-3.61
ages 75+	0-100	1.48	0.63-3.84

Specimen Collection

The blood spot module was randomized to 5/6 of the total NSHAP sample (N=2494), of these, 84.5% (2,105) participated. There were no significant differences between those who agreed to provide blood spots and those who did not with respect to gender, race, ethnicity, age, education, income level, marital status, self-reported mental or physical health, or the reported number of doctor's visits. Due to collection difficulties, blood spots

were not collected from an additional 57 individuals who originally agreed to participate. In total, dried blood spots were collected from 2,048 individuals. From these, 1940 CRP values were obtained.

Blood was obtained from free-flowing capillary blood via a single fingerstick using a retractable-tip, single-use disposable lancet. NSHAP acquired two lancets (model details in Product Availability section below). The Surgilance lancet was used routinely, contained a 2.3 mm blade and was slightly less penetrative. The BD lancet, which contains a slightly larger blade and is commonly used for newborn heel-sticks, was used for cases of thick calluses or where the fingerstick specimen was difficult to obtain with the Surgilance. The blood was allowed to pool on the respondents' finger and four drops of blood were dropped onto filter paper, the first drop being reserved for future genetic analysis. Overall, the procedure required approximately 8 minutes.

Interviewer instructions

- Angle Respondent's hand below their lap.
- Warm finger and stimulate circulation by gently kneading and squeezing the appropriate finger.
- Ask Respondent to gently shake their hand a few times.
- Wipe the index finger of the right hand with alcohol swab and wait a few seconds for the alcohol to dry (DO NOT blow on finger, wave hand, etc. to speed up drying).
- Squeeze the finger just below the area to be pricked.
- Firmly prick finger in the fleshy part of the pad, just off the center.
- IMMEDIATELY dispose of the lancet into the sharps container.
- Allow blood to well on tip of finger.
- If necessary, apply gentle pressure below the site of the prick
- Place first drop in discard circle of filter paper – marked D
- Place 3 (if possible) additional drops on filter paper.
- If unable to fill 3 spots (+ discard spot): Prick another finger. Place first drop in discard circle of filter paper (marked D) and place additional drop(s) in remaining circles.
- If necessary: Ask Respondent to hold cotton ball on finger and apply pressure until bleeding stops.
- Offer Respondent a bandage.
- Label filter paper with su_id.
- Fill out blood spot collection form.
- Place filter paper in plastic bag with desiccant and seal.
- Store at 4^{o}C until shipment.

The filter paper was allowed to dry for the remainder of the interview before being placed in a plastic bag with desiccant for transportation.

Storage and Shipping

After the interview, the filter paper was placed in a Ziploc bag. Once the field investigator reached home, the filter paper was removed from the Ziploc bag and placed in a clear plastic container with the filter paper cover flipped up. A desiccant pack was placed in the container which was then left overnight at room temperature to ensure drying of the

blood spots. In the morning, the filter paper was stored in a sealed Ziploc bag along with the desiccant pack and placed in a storage container. The storage container was kept in the refrigerator at 4°C until shipping day. Blood spots were shipped at room temperature to the designated location. Upon arrival at the laboratory, the specimens were catalogued, analyzed for quality and quantity, and stored at -25°C until analysis.

Shipping Address	Thomas McDade, PhD Northwestern University Department of Anthropology Laboratory for Human Biology Research 1810 Hinman Ave. Evanston, IL 60208

Assay

On day 1, samples were removed from the freezer. Samples were on the benchtop for about 30 minutes for during assay set-up. One 3.2 mm punch was removed from each sample and placed in buffer to elute overnight at 4°C. On day 2, eluate was transferred to a 96 well plate for quantification of CRP using an enzyme immunoassay protocol previously validated for use with dried blood spot samples (McDade et al. 2004). All analyses were conducted at the Laboratory for Human Biology Research at Northwestern University (Evanston, IL).

Samples were analyzed in this order: EBV, CRP, hemoglobin. Samples were sent to Flexsite (Palm City, FL) for a hemoglobin A1c assay soon after receipt in the McDade lab, and prior to EBV analysis.

Table 6. NSHAP Blood Spot CRP testing

Units	Highest Calibrator*	Lowest Calibrator*	Lower limit of sensitivity
mg/L	10.1	0.08	0.028

Scoring

Values reported in milligrams per liter (mg/L). Assay range ≥ 1 mg/L.

Performance Characteristics

A. Precision

Inter-assay precision was established for 10 determinations across 10 assays performed on different days.

Table 7. Intra-Assay Precision

Sample	N	Mean (mg/L)	Coefficient of Variation (%)
Day A			
High	10	5.09	6.4
Low	10	1.01	5.1
Day B			
High	10	5.09	6.9
Low	10	1.01	9.5

B. Sensitivity

The detection limit was defined as the concentration corresponding to the absorbance 2 SD's above the mean of 10 replicates of the 0.00 mg/L calibrator. The lower detection limit of CRP was determined to 0.028 mg/L (McDade et al., 2004), qualifying this dried blood spot method as high-sensitivity.

C. Correlation with Serum

Since serum samples were not collected from respondents in the NSHAP study, a formula showing the correlation between the CRP concentration in blood spot and serum cannot be determined. However, a previous validation study reports the correlation between dried blood spots and serum C-reactive protein by assaying 84 matched samples (McDade et al., 2004). A high correlation was found, R=0.96. The conversion equation from blood spot concentration to serum concentration for this CRP assay is:

****Serum CRP (mg/L)= 1.15*blood spot CRP (mg/L) + 0.13**

******This conversion formula should be used with caution, since the relationship between serum and blood spot CRP concentrations may vary across populations and laboratory methods.

D. Recovery

Control materials with known concentrations of CRP were diluted 1:2 with washed red blood cells. Observed CRP concentration values for low and high controls were 102% and 93% of expected, respectively (McDade et al., 2004).

E. Linearity of Dilution

Linearity of dilution was determined by serially diluting two samples after elution. The range of the observed values was 94.5% to 109% of expected with a mean of 103% (McDade et al., 2004).

Quality Control

Blood spots were obtained on a card containing five pre-printed circles of standard size (1/2 inch/12.7mm diameter). Excellent samples filled the entire circle. Good samples filled a large (6.0mm) hole-punch. Poor samples were ones that did not fill a small (3.2mm) hole punch. Cards were adequate if they allowed for one large and three small punches and excellent if they allowed for more. Throughout the study, the number, quality and condition of the blood spots was recorded by personnel, and if consistent problems were observed from a single field interviewer, they were contacted to discuss problems and techniques to improve the quality of blood spot collection. (Williams & McDade, 2007).

Four control samples were included with each assay to monitor between-assay variation.

Table 7. C-reactive protein quality control outcomes

	Control 1	**Control 2**	**Control 3**	**Control 4**
Average CRP (mg/L)	0.41	1.17	4.32	5.65
SD	0.04	0.09	0.38	0.62
CV	0.099	0.078	0.089	0.110

Product Availability

Product Name	Human Serum C-Reactive Protein (CRP) Calibrator
Manufacturer	Dako
Location of Manufacturer	6392 Via Real Carpinteria, CA 93013 Tel: +1 805 566 6655 Fax: +1 805 566 6688
Catalogue No.	X0923
Product Name	Capture antibody
Manufacturer	Dako
Location of Manufacturer	6392 Via Real Carpinteria, CA 93013 Tel: +1 805 566 6655 Fax: +1 805 566 6688
Catalogue No.	A0073
Product Name	Detection antibody
Manufacturer	Dako
Location of Manufacturer	6392 Via Real Carpinteria, CA 93013 Tel: +1 805 566 6655 Fax: +1 805 566 6688

Catalogue No.	P227
Product Name	OPD Tablets
Manufacturer	Dako
Location of Manufacturer	6392 Via Real Carpinteria, CA 93013 Tel: +1 805 566 6655 Fax: +1 805 566 6688
Catalogue No.	S2045
Product Name	Immuno 96 MicroWell™ Plates
Manufacturer	Nunc Maxisorp
Location of Manufacturer	75 Panorama Creek Drive Rochester, NY 14625-2385 Tel: +1 585 586 8800 Fax: +1 585 586 8987
Catalogue No.	439454
Product Name	SUD-CHEMIE Performance Packaging ; SORB-IT Harmless Absorbant
Manufacturer	Süd-Chemie Performance Packaging
Location of Manufacturer	926 S. 8th St. PO Box 610 Colton, CA 92324 Tel: +1 909 825 1793 +1 800 966 1793 Fax: +1 909 825 6271
Catalogue No.	n/a
Product Name	SurgiLance Safety Lancet, 2.3 mm (blue)
Manufacturer	SurgiLance, Inc.
Location of Manufacturer	Norcross, GA Tel: +1 (770) 448 9493 (US) Fax: + 1 (877) 804 5240 (Toll Free) (US)
Catalogue No.	SLB250
Product Name	BD Quikheel™ Preemie Lancet (pink)
Manufacturer	Becton, Dickinson and Company
Location of Manufacturer	Franklin Lakes, NJ
Catalogue No.	368100

Sources

Procedure developed December 2004 from recommendations by Stacy T. Lindau, University of Chicago, Thomas McDade, Northwestern University and Sharon Williams, Northwestern University. Assay developed by Thomas McDade at Northwestern University Laboratory for Human Biology Research (currently unpublished).

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Epstein-Barr Virus (EBV)

Blood Spot Measurement of Epstein-Barr Virus (EBV) Antibody Titers in Wave I of the National Social Life Health & Aging Project

Authors:
Andreea Mihai, University of Chicago, Department of Ob/Gyn

Thomas McDade, Northwestern University, Departments of Anthropology and Cells to Society (C2S)

Sharon Williams, Purdue University, Department of Anthropology

Stacy Tessler Lindau, MD, MAPP, University of Chicago, Departments of Ob/Gyn and Medicine – Geriatrics, Chicago Core on Biomeasures in Population-Based Health and Aging Research at NORC and the University of Chicago Center on Demography and Economics of Aging*

* Corresponding author.
E-mail address: slindau@uchicago.edu (S.T. Lindau).
Fax: +1 773 834 5664.

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Rationale

Epstein-Barr Virus (EBV) is a member of the herpes virus family, and it typically infects B-lymphocytes(Crawford 2001). It is usually latently expressed(Klein, Kis et al. 2007).

EBV infects about 90% of the world's population(Macsween and Crawford 2003). Infection is lifelong(Callan 2004). While most individuals remain asymptomatic, EBV has been known to contribute to several diseases, including infectious mononucleosis and Burkitt's lymphoma(Macsween and Crawford 2003).

Elevated levels of EBV antibodies are indicative of lower cell-mediated immune function(Glaser, Kiecoltglaser et al. 1985), which has been linked to psychosocial stress(Kiecoltglaser, Garner et al. 1984; Glaser, Kiecoltglaser et al. 1985). Lower cell-mediated immune function increases an individual's risk for B-cell lymphoproliferative disease(Crawford 2001). Additionally, those who test negative for EBV antibodies are at an increased risk for severe EBV-associated diseases if they become infected in later life(Crawford 2001). Compromised immunity due to any factor (including immunosenescence)(Srinivasan, Maestroni et al. 2005) also increases the risk for pathological EBV infection(Bennett 2007).

NSHAP's in-home biological data collection design was based on the principles of minimal invasiveness(Lindau and McDade 2007). Specimens for EBV antibody titer assays were collected in the form of dried blood spots; this is a minimally invasive process that can be safely carried out in the home by a trained lay interviewer(McDade, Williams et al. 2007). This method has been successfully implemented in a variety of studies in the US in addition to NSHAP (e.g. the Health and Retirement Study and the National Longitudinal Study of Adolescent Health) and internationally, including lowland Bolivia, Samoa, Kenya, Papua New Guinea, and Nepal(Worthman and Stallings 1997; McDade, Stallings et al. 2000b; Panter-Brick, Lunn et al. 2001; Shell-Duncan and McDade 2004; McDade, Leonard et al. 2005).

Measurement

The standard assay for clinical EBV detection involves indirect immunofluorescence using blood plasma(McDade, Stallings et al. 2000a). In NSHAP, blood was collected by finger stick using a disposable lancet and then captured on filter paper for transport and storage. (Details about collection equipment are described in the Availability section, below.) To determine EBV IgG antibody titers, a measure of EBV infection(McDade, Stallings et al. 2000a), the ELISA (enzyme-linked immunosorbent assay) assay was used on a 3.2 or 6.0 mm sample from the filter paper blood spot specimen. Previous research has established that the ELISA assay produces comparable results with indirect immunofluorescence, with a strong positive correlation between the two

(Pearson R = 0.77; p<0.01)(McDade, Stallings et al. 2000a). The correlation between EBV antibody titers obtained from blood plasma and blood spots is also high (R = 0.936)(McDade, Stallings et al. 2000a).

The ELISA assay measures IgG antibodies against the p18 viral capsid antigen (VCA) complex. The dried blood spot protocol is an adaptation of a commercially available kit designed for use with serum or plasma (DiaSorin Corporation, Stillwater, MN, product no. P001606A)(McDade, Stallings et al. 2000a).

Population Norms

Early exposure to the virus is more prevalent in developing countries, where the rate of infection approaches 100% during the first 5 years of life(McDade, Stallings et al. 2000b). Related pathology is less likely to occur if a person is exposed to the virus early(Crawford 2001).

In industrialized nations, 25-50% of adolescents are infected with EBV by the age of 18. By the age of 40, 80-90% of adults are infected(Jones and Strauss 1987).

Each individual's viral load remains fairly constant over time (about 5-500 virus infected cells in every $10x10^6$ circulating B cells)(Crawford 2001). Qualitative descriptions (low level of EBV antibodies, high level, etc) are matched with their quantitative measurements in Table 1. Because older adults are relatively immunosupressed, their EBV antibody levels are expected to be in the mid-high to high range.

Most research on EBV is based on populations with EBV-linked pathology. Normative data on EBV antibody titers have been described among younger adults under specialized conditions – ie, geographical location or infection of certain diseases(McDade, Stallings et al. 2000a; McDade, Stallings et al. 2000b; Shimoyama, Yamamoto et al. 2008). However, because both EBV-specific assays and data interpretations are not standardized, comparisons across studies should be interpreted cautiously(Hess 2004). Higher titers are associated with lower cell-mediated immune function; lower titers are associated with higher cell-mediated immune function (Glaser, Kiecoltglaser et al. 1985). Normative data on older adults without EBV-linked pathology, to the best of our knowledge, have not been previously generated.

Specimen Collection

The blood spot module was randomized to 5/6 of the total NSHAP sample (N=2494), of these, 84.5% (2,105) participated. There were no significant differences between those who agreed to provide blood spots and those who did not with respect to gender, race, ethnicity, age, education, income level, marital status, self-reported mental or physical health, or the reported number of doctor's visits. Due to collection difficulties, blood spots were not collected from an additional 57 individuals who originally agreed to participate. In total, dried blood spots were collected from 2,048 individuals. From these, 1981 EBV values were obtained.

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Storage and Shipping

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the specimens were catalogued, analyzed for quality and quantity, and stored at -25^{O}C until analysis.

Shipping Address	Thomas McDade, PhD Northwestern University Department of Anthropology Laboratory for Human Biology Research 1810 Hinman Ave. Evanston, IL 60208

Assay

On day 1, samples were removed from the freezer and one 3.2 mm punch was removed from each sample and placed in buffer to elute overnight. On day 2, eluate was transferred to a 96 well plate for quantification of EBV antibodies using an ELISA protocol previously validated for use with dried blood spot samples (McDade et al. 2000). All analyses were conducted at the Laboratory for Human Biology Research at Northwestern University (Evanston, IL).

Performance Characteristics

A. Precision

Table 1. EBV p18-VCA Antibody Assay Precision (Within-Assay Coefficient of Variation) and Reliability (Between-Assay Coefficient of Variation)[a]

Control Level	Within-Assay (N=10 Determinations)		Between-Assay (N=12 Runs)	
	Mean ± SD	% CV	Mean ± SD	% CV
Low	29.4 ± 2.7	9.2	30.6 ± 4.3	14.1
Mid-low	111.4 ± 3.7	3.3	100.2 ± 5.0	5.0
Mid-high	212.1 ± 11.1	5.3	206.3 ± 8.8	4.3
High	271.7 ± 12.1	4.5	278.3 ± 19.1	6.9

[a]Mean p18-VCA antibody titres are presented in standard ELISA scores.
Reproduced with permission from McDade, Stallings et al. 2000a

B. Sensitivity

Values below 20 ELISA units were assumed to be seronegative. For a discussion of the sensitivity of the blood spot based EBV assay, please see McDade, Stallings et al. 2000b.

C. Correlation with Plasma

The correlation between blood spots and plasma was pre-determined by assaying 40 matched samples. A drop from each whole blood sample (EDTA-anticoagulated) was sampled onto filter paper while the other portion was centrifuged in order to withdraw the plasma. Previous literature determined the correlation to be high with an r value of 0.936(McDade, Stallings et al. 2000a).

The correlation between the standard indirect immunofluorescence technique for EBV analysis and ELISA technique used in NSHAP was likewise high ($r=0.77$, $p<0.01$)(McDade, Stallings et al. 2000a). One hundred eleven assays were matched in the validation study.

Quality Control

Blood spots were obtained on a card containing five pre-printed circles of standard size (1/2 inch/12.7mm diameter). Excellent samples filled the entire circle. Good samples were large enough to provide sample for a large (6.0mm) hole punch. Poor samples were ones that did not provide enough for a single (3.2mm) hole punch. Cards were adequate if they allowed for one large and three small punches and excellent if they allowed for more. Throughout the study, the number, quality and condition of the blood spots was recorded by personnel, and if consistent problems were observed from a single field interviewer, they were contacted to discuss problems and techniques to improve the quality of blood spot collection(Williams and McDade 2007).

Four blood spot control samples were included with each assay to monitor between-assay variation.

Table 2. Between-Assay Variation in Blood Spot Control Samples

	1	**2**	**3**	**4**
average	25.53	118.60	237.30	286.70
SD	3.42	10.42	12.19	17.01
CV	0.134	0.088	0.051	0.059

Product Availability

Product Name	Epstein-Barr virus VCA IgG ELISA
Manufacturer	DiaSorin, Inc.
Location of Manufacturer	1951 Northwestern Avenue - P.O. Box 285 MN 55082-0285 Stillwater Tel: +1.651.439.9710 +1.800.328.1482 Fax: +1.651.351.5669 (USA & Canada only)
Catalogue No.	P001606A
Product Name	903™ Multiple-part Neonatal Card

Manufacturer	Schleicher & Schuell BioScience
Location of Manufacturer	Keene, NH 03431 Contact: Judy Peter 800- 437-7003
Catalogue No.	10 537 279
Product Name	SUD-CHEMIE Performance Packaging; SORB-IT Harmless Absorbant
Manufacturer	Süd-Chemie Performance Packaging
Location of Manufacturer	926 S. 8th St. PO Box 610 Colton, CA 92324 Tel: +1 909 825 1793 +1 800 966 1793 Fax: +1 909 825 6271
Catalogue No.	n/a
Product Name	SurgiLance Safety Lancet, 2.3 mm (blue)
Manufacturer	SurgiLance, Inc.
Location of Manufacturer	Norcross, GA Tel: +1 (770) 448 9493 (US) Fax: + 1 (877) 804 5240 (Toll Free) (US)
Catalogue No.	SLB250
Product Name	BD Quikheel™ Preemie Lancet (pink)
Manufacturer	Becton, Dickinson and Company
Location of Manufacturer	Franklin Lakes, NJ
Catalogue No.	368100

Sources

Procedure developed December 2004 from recommendations by Stacy T. Lindau, University of Chicago, Thomas McDade, Northwestern University and Sharon Williams, Northwestern University. Assay developed by Thomas McDade at Northwestern University Laboratory for Human Biology Research (currently unpublished).

Scoring & Usage

Values are reported in enzyme-linked immunosorbent assay (ELISA) units. Here, they are a measure of the amount of p18-VCA in the blood spot(McDade, Stallings et al. 2000a).

If the sample has less than 20 ELISA units, the subject is seronegative. If the sample has more than 20 ELISA units, the subject is seropositive. The recode category is positive/negative(McDade, Stallings et al. 2000a).

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Glycosylated Hemoglobin (HbA1c)

Dried Blood Spot Measurement of Glycosylated Hemoglobin (HbA1c) in Wave I of the National Social Life Health & Aging Project

Authors:

Ada Gomero, University of Chicago, Department of Ob/Gyn

Thomas McDade, Northwestern University, Departments of Anthropology and Cells to Society (C2S)

Sharon Williams, Purdue University, Department of Anthropology

Stacy Tessler Lindau, MD, MAPP, University of Chicago, Departments of Ob/Gyn and Medicine – Geriatrics, Chicago Core on Biomeasures in Population-Based Health and Aging Research at NORC and the University of Chicago Center on Demography and Economics of Aging *

* Corresponding author.
E-mail address: slindau@uchicago.edu (S.T. Lindau).
Fax: +1 773 834 5664

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URL:

Date: October 8, 2008

Contents

Rationale

HbA1c, or glycosylated hemoglobin, is formed through the non-enzymatic binding of circulating glucose to hemoglobin (glycation). HbA1c is measured as the ratio of glycosylated to nonglycosylated hemoglobin (Peterson, Pavlovich et al. 1998). Higher levels of glucose in the blood contribute to more binding and consequent higher levels of glycosylated hemoglobin.

Glycation occurs over the entire 90-120 day life span of the red blood cell (Kilpatrick 2000). HbA1c can consequently be interpreted as an average of the blood glucose present over the past 3-4 months (Peterson, Pavlovich et al. 1998). Measurement of HbA1c is accepted as a useful index of mean blood glucose in the treatment of patients with diabetes (Rohlfing, Little et al. 2000). Decisions regarding treatment are often based on HbA1c. Although the American Diabetes Association (ADA) does not currently recommend HbA1c measurement for the diagnosis of diabetes, studies have shown frequency distributions similar to those of fasting plasma glucose (FPG) used in diagnosing diabetes (McCance, Hanson et al. 1994; Engelgau, Thompson et al. 1997; 2003). HbA1c is a more comprehensive measure of total glycemic exposure than FPG due to the representation of blood glucose in the postprandial state in addition to the fasting state (Rohlfing, Little et al. 2000).

HbA1c concentration is associated with diabetic microvascular complications (Rohlfing, Little et al. 2000; 2003), macrovascular complications (Khaw, Wareham et al. 2001; 2003), risk of death (Khaw, Wareham et al. 2001), and cardiovascular disease (Khaw, Wareham et al. 2001). Positive correlations with metabolic syndrome are also suggested (Grant, Soriano et al. 2004).

HbA1c <6% is considered normal (2007). The ADA-recommended treatment goal for the diabetic population is HbA1c <7% (2003), although the target may be higher for older adults (2007; Chin, Drum et al. 2008). HbA1c levels as low as 6.2% have been cited as at risk threshold values for development of cardiovascular complications (Rohlfing, Wiedmeyer et al. 2002; Grant, Soriano et al. 2004). Clinical and population studies have found racial, ethnic, and age disparities in HbA1c levels (Hashimoto, Futamura et al. 1995; Gilliland, Carter et al. 2002; Karter, Ferrara et al. 2002; Saaddine, Fagot-Campagna et al. 2002; Kirk, D'Agostino et al. 2006; Kirk, Passmore et al. 2008).

Measurement

Measurement of HbA1c is usually determined using whole blood samples via venipuncture. In NSHAP, blood was collected by finger-stick using a disposable lancet, and then captured on filter paper for transport and storage. Blood spot collection offers a

convenient, minimally invasive alternative to venipuncture (Lindau and McDade 2007; McDade, Williams et al. 2007). Blood spots facilitate in-home sample collection and can be performed by non-medically trained personnel (Williams and McDade 2008). Assay techniques for HbA1c include affinity chromatography, electrophoresis, high performance liquid chromatography (HPLC), and immmunoassays. The "gold standard" of these methods is assay by HPLC (Halwachs-Baumann, Katzensteiner et al. 1997; Ray and Kerestan 2000). For the HbA1c assay used, high correlation of results is reported when compared to clinical standard methods (Flexsite; Williams and McDade 2008).

Interpretation of results from identical HbA1c samples assayed in different laboratories often varies due to absence of comprehensive HbA1c assay result standardization. The National Glycohemoglobin Standardization Program (NGSP) continues to standardize assay results to generalize them to larger studies and populations (Rohlfing, Little et al. 2000). The Roche Unimate method used by the NSHAP study is NGSP-certified.

Population Norms

Table 1 shows an age-dependent increase in HbA1c (Hashimoto, Futamura et al. 1995).

Table 1. Distribution of Mean HbA1c levels (% of total hemoglobin)

Age	Men	Women	Source
5-24 years	5.02%	4.95%	NHANES III; (Saaddine, Fagot-Campagna et al. 2002)
40-45 years	5.02%	4.1%	The Telecom Study; (Simon, Senan et al. 1989)
≥ 60 years	5.05%	5.32%	The Telecom Study; (Simon, Senan et al. 1989)

Although the use of HbA1c to diagnose conditions is not fully standardized, it is a recommended and accepted measure for risk of disease. Prevalence of retinopathy, for instance, is observed to increase sharply at HbA1c levels at or above 6.2% (2003).

Table 2. NSHAP Summary Statistics for HbA1c levels (% of total hemoglobin) (Williams and McDade 2008)

	Range	Mean (weighted) HbA1c (% of total Hb)	Standard Deviation
Men			
ages 57-65	4.70-13.4	6.21	1.39
ages 66-75	4.60-11.4	6.07	0.89
ages 76+	4.20-14.2	6.09	1.02
Women			
ages 57-65	4.50-12.5	5.99	0.99
ages 66-75	4.50-11.4	5.91	0.82
ages 76+	4.60-13.5	6.04	0.82

Specimen Collection

The blood spot module was randomized to 5/6 of the total NSHAP sample (N=2494), of these, 84.5% (2,105) participated. There were no significant differences between those who agreed to provide blood spots and those who did not with respect to gender, race, ethnicity, age, education, income level, marital status, self-reported mental or physical health, or the reported number of doctor's visits. Due to collection difficulties, blood spots were not collected from an additional 57 individuals who originally agreed to participate. In total, dried blood spots were collected from 2,048 individuals. From these, 1739 HbA1c analytes were obtained.

Blood was obtained from free-flowing capillary blood via a fingerstick with a retractable-tip disposable lancet. NSHAP acquired two lancets (model details in Product Availability section below). The Surgilance lancet was used routinely, contained a 2.3 mm blade and was slightly less penetrative. The BD lancet, which contains a slightly larger blade and is commonly used for newborn heel-sticks, was used for cases of thick calluses or where the fingerstick specimen was difficult to obtain with the Surgilance. The blood was allowed to pool on the respondents' finger and four drops of blood were dropped onto filter paper, the first drop being reserved for future genetic analysis. Overall, the procedure required approximately 8 minutes.

Interviewer instructions

- Angle Respondent's hand below their lap.
- Warm finger and stimulate circulation by gently kneading and squeezing the appropriate finger.
- Ask Respondent to gently shake their hand a few times.
- Wipe the index finger of the right hand with alcohol swab and wait a few seconds for the alcohol to dry (DO NOT blow on finger, wave hand, etc. to speed up drying).
- Squeeze the finger just below the area to be pricked.
- Firmly prick finger in the fleshy part of the pad, just off the center.
- IMMEDIATELY dispose of the lancet into the sharps container.

- Allow blood to well on tip of finger.
- If necessary, apply gentle pressure below the site of the prick
- Place first drop in discard circle of filter paper – marked D
- Place 3 (if possible) additional drops on filter paper.
- If unable to fill 3 spots (+ discard spot): Prick another finger. Place first drop in discard circle of filter paper (marked D) and place additional drop(s) in remaining circles.
- If necessary: Ask Respondent to hold cotton ball on finger and apply pressure until bleeding stops.
- Offer Respondent a bandage.
- Label filter paper with su_id.
- Fill out blood spot collection form.
- Place filter paper in plastic bag with desiccant and seal.
- Store at 4^{o}C until shipment.

The filter paper was allowed to dry for the remainder of the interview before being placed in a plastic bag with desiccant for transportation.

Storage and Shipping

After the interview, the filter paper was placed in a Ziploc bag. Once the field investigator reached home, the filter paper was removed from the Ziploc bag and placed in a clear plastic container with the filter paper cover flipped up. A desiccant pack was placed in the container which was then sealed overnight and left at room temperature to ensure drying of the blood spots. In the morning, the filter paper was stored in a sealed Ziploc bag along with the desiccant pack and placed in a storage container. The storage container was kept in the refrigerator at 4°C until shipping day. Blood spots were shipped at room temperature to the designated location. Upon arrival at the laboratory, the filter papers were catalogued and frozen at -25°C until analysis.

Shipping Address	Thomas McDade, PhD Northwestern University Associate Professor Department of Anthropology Laboratory for Human Biology Research 1810 Hinman Ave. Evanston, IL 60208

Blood spots to be assayed for HbA1c were then sent to a central analytical laboratory.

Laboratory Shipping Address	Flexsite Diagnostics, Inc. 3541 SW Corporate Pkwy Palm City, FL 34990 Contact: Robert Ray 1 772 221 8893

Assay

NSHAP used blood spot filter paper collection of capillary blood for assay of HbA1c. One spot on the filter paper from Flexsite Diagnostics was pretreated with a proprietary solution to facilitate re-hydration of the dried blood prior to analysis. At the laboratory, blood was eluted from the blood spot on the filter paper. The Roche Unimate immunoassay and Cobas Integra Analyzer were used to perform the assay. The immunoassay incorporates a latex-enhanced competitive turbidimetric immunoassay, which determines HbA1c concentration, with a colorimetric quantification of total hemoglobin. The Cobas Integra analyzer, which is NGSP-certified, is calibrated using a synthetic HbA1c standard (Ray and Kerestan 2000).

Performance Characteristics

A. Precision

Precision for this assay was determined by assaying two normal blood spots and two abnormal (elevated) blood spots twice daily for twenty days, following the National Committee for Clinical Laboratory Standards procedure EP-5A (Ray and Kerestan 2000). The American Diabetes Association recommends a CV < 5%.

Table 3. Typical precision for HbA1c assay with normal and abnormal blood spots on treated filter paper (stored at -70ºC)

	Normal	**Elevated**
Within Run CV	0.88%	1.71%
Between Run CV	1.31%	1.90%
Total (20 day) CV	1.46%	2.54%
Reproduced with permission from Robert Ray, Flexsite Diagnostics		

B. Accuracy

Accuracy was determined by comparing physician-collected dried blood spot samples to venipuncture whole blood hemolysate samples. Correlation of the blood spot HbA1c obtained from the doctor's office blood specimen with the sample obtained from the venipuncture specimen was r=0.978 (Ray and Kerestan 2000).

C. Correlation with Serum

The correlation of home-collected blood spots (from same patients) with whole blood was r=0.956. Assay results are highly correlated with the standard Roche Unimate immunoassay on whole blood (Ray and Kerestan 2000).

D. Comparison with reference technology Diamat HPLC

Correlation of this method with HPLC (r=0.981) indicates that results are highly accurate relative to the "gold standard" method (Ray and Kerestan 2000).

Quality Control

Blood spots were obtained on a card containing five pre-printed circles of standard size (1/2 inch/12.7mm diameter). Excellent samples filled the entire circle. Good samples filled a large (6.0mm) hole-punch. Poor samples were ones that did not fill a small (3.2mm) hole punch. Cards were adequate if they allowed for one large and three small punches and excellent if they allowed for more. Throughout the study, the number, quality and condition of the blood spots was recorded by personnel, and if consistent problems were observed from a single field interviewer, they were contacted to discuss problems and techniques to improve the quality of blood spot collection. (Williams and McDade, 2007).

The stability of HbA1c in dried blood spots for this assay under typical mailing conditions was assessed by comparing blood spots on filter paper to whole blood hemolysate values at room temperature at 1, 7, and 14 days from collection. Correlation coefficients at each time period were $r > 0.960$, demonstrating high stability of the blood spot analyte at room temperature. A minimal slow rate of glycation was observed in the dry state samples over two weeks. Only minimal losses of glycation, which do not detract from the value of this technique, were observed in assessments of different mailing conditions. Further, no significant effect on the samples exists from multiple cycles of freezing and thawing, demonstrating the excellent stability of dried blood spots (Ray and Kerestan 2000).

Scoring & Usage

The NSHAP dried blood spots sample size for HbA1c was 1739. Results are reported as percent of glycosylated hemoglobin (of total hemoglobin) (Williams and McDade 2008).

Product Availability

Product Name	A1c At-Home
Manufacturer	Flexsite Diagnostics,Inc.
Location of Manufacturer	Palm City, FL Contact: Robert Ray 1 772 221 8893
Catalogue No.	Custom Product
Product Name	903™ Multiple-part Neonatal Card
Manufacturer	Schleicher & Schuell BioScience
Location of Manufacturer	Keene, NH 03431 Contact: Judy Peter 800- 437-7003
Catalogue No.	10 537 279
Product Name	SurgiLance Safety Lancet, 2.3 mm (blue)
Manufacturer	SurgiLance, Inc.
Location of Manufacturer	Norcross, GA Tel: +1 (770) 448 9493 (US) Fax: + 1 (877) 804 5240 (Toll Free) (US)

Catalogue No.	SLB250
Product Name	BD Quikheel™ Preemie Lancet (pink)
Manufacturer	Becton, Dickinson and Company
Location of Manufacturer	Franklin Lakes, NJ
Catalogue No.	368100

Sources

Procedure developed December 2004 from recommendations by Stacy T. Lindau, University of Chicago, Thomas McDade, Northwestern University and Sharon Williams, Northwestern University. Assay developed by Thomas McDade at Northwestern University Laboratory for Human Biology Research (currently unpublished).

Acknowledgement

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Hemoglobin

Blood Spot Measurement of Hemoglobin in Wave I of the National Social Life Health & Aging Project

Authors:

Alina Fomovska, University of Chicago, Department of Ob/Gyn

Thomas McDade, Northwestern University, Departments of Anthropology and Cells to Society (C2S)

Sharon Williams, Purdue University, Department of Anthropology

Stacy Tessler Lindau, MD, MAPP, University of Chicago, Departments of Ob/Gyn and Medicine – Geriatrics, Chicago Core on Biomeasures in Population-Based Health and Aging Research at NORC and the University of Chicago Center on Demography and Economics of Aging *

* Corresponding author.
E-mail address: slindau@uchicago.edu (S.T. Lindau).
Fax: +1 773 834 5664

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Date: October 10, 2008

Contents

Rationale

Hemoglobin is the red blood cell protein that carries oxygen in the blood. Anemia is an unhealthy condition indicated by low hemoglobin levels, and can ultimately be the result of iron, folate or B_{12} deficiency, renal insufficiency, chronic inflammation, acute or chronic bleeding, or other causes. About a third of cases of anemia among older individuals is caused by iron, folate, or B_{12} deficiency. Other causes include chronic inflammation (19.7%), chronic renal failure (8.2%) or both (4.3%). Another third of cases are unexplained(Guralnik, Eisenstaedt et al. 2004).

Anemia is associated with an increased risk of cardiovascular disease, cognitive dysfunction, reduced bone density, longer hospitalization for elective procedures(Eisenstaedt, Pennix et al. 2006), as well poor outcomes in many chronic diseases(Goodnough and Nissenson 2004). Among the older population, anemia can be an independent risk factor for death(Lipschitz 2003). Anemia is also associated with fatigue and decline in physical performance, which may lead to a loss of independence and other social and economic effects(Penninx, Guralnik et al. 2003). As such, anemia can have a significant effect on the quality of life of older individuals(Eisenstaedt, Pennix et al. 2006). The prevalence of anemia increases with age(Guralnik, Eisenstaedt et al. 2004). Estimated prevalence rates for older populations in the United States vary widely from 3.9% - 59.9% depending on the population studied and criteria used for defining anemia(Beghe, Wilson et al. 2004). Some studies suggest that even “low normal” hemoglobin levels may be associated with poor performance on mobility tests and physical function(Eisenstaedt, Pennix et al. 2006).

In healthy older individuals between 60 and 98 years of age, hemoglobin levels do not change significantly(Balducci 2003). Normal range of hemoglobin levels is defined by the World Health Organization as between 12 and 16 g/dL. By WHO criteria, anemia is defined as a hemoglobin concentration lower than 13 g/dL in men and lower than 12 g/dL in women. Severe anemia is characterized as Hb<10 g/dL(Charves et al. 2004).

Measurement

In NSHAP, blood was collected by finger-stick using a disposable lancet, and then captured on filter paper for transport and storage. (Details about collection equipment are described in the “Specimen Collection” section, below.)

NSHAP’s in-home biological data collection design was based on the principles of minimal invasiveness(Lindau and McDade 2007). For this reason, blood was collected by a finger-stick using a disposable lancet (as opposed to venipuncture), and then captured on filter paper for transport and storage.

Population Norms

Table 1. Prevalence of anemia, 65 and older in the U.S., by gender

Race/ethnic group	Men	Women
Non-Hispanic/white	9.2 %	8.7 %
Non-Hispanic/black	27.5 %	28.0 %
Mexican American	11.5 %	9.3 %
Other	20.4	7.5
Total	11.0	10.2

(Guralnik, Eisenstaedt et al. 2004)

Specimen Collection

The blood spot module was randomized to 5/6 of the total NSHAP sample (N=2494), of these, 84.5% (2,105) participated. There were no significant differences between those who agreed to provide blood spots and those who did not with respect to gender, race, ethnicity, age, education, income level, marital status, self-reported mental or physical health, or the reported number of doctor's visits. Due to collection difficulties, blood spots were not collected from an additional 57 individuals who originally agreed to participate. In total, dried blood spots were collected from 2,048 individuals. From these, 1940 CRP values were obtained.

Blood was obtained from free-flowing capillary blood via a single fingerstick using a retractable-tip, single-use disposable lancet. NSHAP acquired two lancets (model details in Product Availability section below). The Surgilance lancet was used routinely, contained a 2.3 mm blade and was slightly less penetrative. The BD lancet, which contains a slightly larger blade and is commonly used for newborn heel-sticks, was used for cases of thick calluses or where the fingerstick specimen was difficult to obtain with the Surgilance. The blood was allowed to pool on the respondents' finger and four drops of blood were dropped onto filter paper, the first drop being reserved for future genetic analysis. Overall, the procedure required approximately 8 minutes.

Interviewer instructions

- Angle Respondent's hand below their lap.
- Warm finger and stimulate circulation by gently kneading and squeezing the appropriate finger.
- Ask Respondent to gently shake their hand a few times.
- Wipe the index finger of the right hand with alcohol swab and wait a few seconds for the alcohol to dry (DO NOT blow on finger, wave hand, etc. to speed up drying).

- Squeeze the finger just below the area to be pricked.
- Firmly prick finger in the fleshy part of the pad, just off the center.
- IMMEDIATELY dispose of the lancet into the sharps container.
- Allow blood to well on tip of finger.
- If necessary, apply gentle pressure below the site of the prick
- Place first drop in discard circle of filter paper – marked D
- Place 3 (if possible) additional drops on filter paper.
- If unable to fill 3 spots (+ discard spot): Prick another finger. Place first drop in discard circle of filter paper (marked D) and place additional drop(s) in remaining circles.
- If necessary: Ask Respondent to hold cotton ball on finger and apply pressure until bleeding stops.
- Offer Respondent a bandage.
- Label filter paper with su_id.
- Fill out blood spot collection form.
- Place filter paper in plastic bag with desiccant and seal.
- Store at 4^{o}C until shipment.

The filter paper was allowed to dry for the remainder of the interview before being placed in a plastic bag with desiccant for transportation.

Storage and Shipping

After the interview, the filter paper was placed in a Ziploc bag. Once the field investigator reached home, the filter paper was removed from the Ziploc bag and placed in a clear plastic container with the filter paper cover flipped up. A desiccant pack was placed in the container which was then sealed overnight and left at room temperature to ensure drying of the blood spots. In the morning, the filter paper was stored in a sealed Ziploc bag along with the desiccant pack and placed in a storage container. The storage container was kept in the refrigerator at 4°C until shipping day. Blood spots were shipped at room temperature to the designated location. Upon arrival at the laboratory, the specimens were catalogued, analyzed for quality and quantity, and stored at -25^{O}C until analysis.

Shipping Address	Thomas McDade, PhD Northwestern University Department of Anthropology Laboratory for Human Biology Research 1810 Hinman Ave. Evanston, IL 60208

Assay

The assay is a colorimetric method based on the positive and highly linear relationship between level of hemoglobin in the sample and amount of color development that occurs after reaction with a single reagent, Drabkin's solution. All hemoglobins in the sample (except sulfhemoglobin) are converted to cyanmethemoglobin which can be read photometrically at 540 nM. Potassium cyanide in Drabkin's reagent converts Hb iron from the ferrous to the ferric state to form methemoglobin. Methemoglobin combines with potassium cyanide (also in Drabkin's solution) to produce the stable and easily measurable cyanmethemoglobin. Because the color development is directly proportional to the concentration, unknown samples are easily quantified by comparison to known concentrations in a standard curve. A single disc of blood was punched from each sample using 3.2mm hole punch.

Table 2. NSHAP Summary statistics for hemoglobin levels (g/dL)

Hemoglobin (mg/dL)	Range	Mean (weighted)	Standard Deviation
Men			
Ages 57-65	8.89-18.5	13.8	1.76
Ages 66-75	7.65-18.7	13.2	1.92
Ages 76+	7.56-17.0	12.6	1.72
Women			
Ages 57-65	7.89-18.3	12.6	1.63
Ages 66-75	4.50-18.4	12.3	1.81
Ages 76+	6.58-16.4	12.0	1.66

Table 3 NSHAP Blood Spot Hemoglobin Testing Performed at Northwestern University

Units	Highest Calibrator*	Lowest Calibrator*	Lower limit of sensitivity
g/dL	8	18.9	NA

(Williams and McDade 2007)

Scoring & Usage

- Results are reported in g/dL (grams per deciliter).
- Anemia is defined as <12 g/dL in women and 13 g/dL in men based on WHO(World Health Organization 1968).

Quality Control

Blood spots were obtained on a card containing five pre-printed circles of standard size (1/2 inch/12.7mm diameter). Excellent samples filled the entire circle. Good samples filled a large (6.0mm) hole-punch. Poor samples were ones that did not fill a small (3.2mm) hole punch. Cards were adequate if they allowed for one large and three small punches and excellent if they allowed for more. Throughout the study,

the number, quality and condition of the blood spots was recorded by personnel, and if consistent problems were observed from a single field interviewer, they were contacted to discuss problems and techniques to improve the quality of blood spot collection (Williams and McDade 2007).

Three control samples were used for estimating between-assay variation:

Average	6.764286	13.74446	22.47839
Standard deviation	0.523954	0.655389	1.402853
% coefficient of variation	0.077459	0.047684	0.062409

Product Availability

Product Name	903™ Multiple-part Neonatal Card
Manufacturer	Schleicher & Schuell BioScience
Location of Manufacturer	Keene, NH 03431 Contact: Judy Peter 800- 437-7003
Catalogue No.	10 537 279
Product Name	SUD-CHEMIE Performance Packaging ; SORB-IT Harmless Absorbant
Manufacturer	Süd-Chemie Performance Packaging
Location of Manufacturer	926 S. 8th St. PO Box 610 Colton, CA 92324 Tel: +1 909 825 1793 +1 800 966 1793 Fax: +1 909 825 6271
Catalogue No.	n/a
Product Name	SurgiLance Safety Lancet, 2.3 mm (blue)
Manufacturer	SurgiLance, Inc.
Location of Manufacturer	Norcross, GA Tel: +1 (770) 448 9493 (US) Fax: + 1 (877) 804 5240 (Toll Free) (US)
Catalogue No.	SLB250
Product Name	BD Quikheel™ Preemie Lancet (pink)
Manufacturer	Becton, Dickinson and Company
Location of Manufacturer	Franklin Lakes, NJ
Catalogue No.	368100

Sources

Procedure developed December 2004 from recommendations by Stacy T. Lindau, University of Chicago, Thomas McDade, Northwestern University and Sharon Williams, Northwestern University. Assay developed by Thomas McDade at Northwestern University Laboratory for Human Biology Research (currently unpublished).

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