Innovative Non- or Minimally-Invasive Technologies for Monitoring Health and Nutritional Status in Mothers and Young Children

Use of Filter Paper for the Collection and Analysis of Human Whole Blood Specimens[1]

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ABSTRACT The Centers for Disease Control and Prevention and its partners have been operating the Newborn Screening Quality Assurance Program for >20 y. The program helps participating laboratories to evaluate and improve the quality of their newborn-screening testing efforts by providing quality control dried blood spot materials and proficiency-testing materials for the external evaluation of screening programs. The Newborn Screening Quality Assurance Program provides an independent evaluation of filter papers approved by the Food and Drug Administration for the collection of blood for clinical tests. These activities have created a mechanism for the validation of the filter paper blood collection device and the standardization of materials and methods for the analysis of dried blood spots. J. Nutr. 131: 1631S–1636S, 2001.

KEY WORDS: • *newborn screening* • *dried blood spots* • *blood collection* • *quality assurance*

The use of filter paper for the collection and analysis of human blood dates back to the early 1960s, when Dr. Robert Guthrie used dried blood spot specimens to measure phenylalanine in newborns for the detection of phenylketonuria (Guthrie and Susi 1963). This novel application for collecting blood led to the population screening of newborns for the detection of treatable, inherited metabolic diseases. Today, state public health laboratories screen >95% of all newborns in the largest genetic testing effort in the United States. Filter paper has been used to collect blood for public health purposes for almost 40 y. The paper is made from high purity cotton linters and is manufactured to give accurate and reproducible absorption of blood specimens according to National Committee on Clinical Laboratory Standards (NCCLS)[3] specifications (Hannon et al. 1997). The filter paper blood collection device has achieved the same level of precision and reproducibility that analytical scientists and clinicians have come to expect from standard methods of collecting blood, such as vacuum tubes and capillary pipettes. Like these devices, filter paper has associated with it some level of imprecision that can be characterized to standardize the device and to minimize the variation in measurements due to the filter paper matrix. In addition, the ease of collection, transport and storage make dried blood spot specimens a cost-effective choice for many clinical applications. Here we describe the efforts of the Newborn Screening Quality Assurance Program (NSQAP) at the Centers for Disease Control and Prevention (CDC) to provide external quality assurance for the filter paper blood collection device, for newborn-screening laboratories and for epidemiological research using dried blood spots.

Quality assurance for newborn-screening tests

In collaboration with its partners, the NSQAP at the CDC has served, for over 20 years, the laboratories that use dried blood spot specimens for newborn-screening tests by providing technical assistance, consultation and training for laboratory methods, evaluation and distribution of reference and quality control dried blood spot materials, a mechanism for the voluntary evaluation of laboratory performance (proficiency testing), the transfer of new technologies and the external evaluation of programs that screen for hypothyroidism, phenylketonuria, other inborn errors of metabolism and hemoglobinopathies (Hannon et al. 2000). At this writing, NSQAP provides quality control materials, proficiency-testing services and technical support to 216 laboratories in 37 countries worldwide. These laboratories include domestic screening laboratories and manufacturers of diagnostic products. Our list of

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[3] Abbreviations used: NCCLS, National Committee on Clinical Laboratory Standards; NSQAP, Newborn Screening Quality Assurance Program; CDC, Centers for Disease Control and Prevention; DNA, deoxyribonucleic acid; HIV, human immunodeficiency virus.

participants grows every year as more countries adopt newborn-screening programs for their populations.

NSQAP provides screening laboratories with both known quality control dried blood spot materials for routine use and proficiency-testing materials that consist of blind-coded specimens for laboratory performance evaluation. Quality control materials are used on a routine basis and with the laboratories' screening methods and enable participating laboratories to achieve high levels of technical proficiency and continuity. The program distributes quality control materials for thyroxin, thyroid stimulating hormone, phenylalanine, galactose, 17-α-hydroxyprogesterone, luecine and methionine. These materials perform consistently in assays despite changes in commercial reagents. For proficiency testing, panels of five blind-coded dried blood spot specimens are distributed for these analytes, in addition to separate panels prepared with purchased blood from donors with biotinidase deficiency and galactose-1-phosphate uridyltransferase deficiency. For hemoglobinopathies, panels are prepared from umbilical cord blood. Quarterly reports summarizing all proficiency-testing data (quantitative and qualitative) received by the cutoff date are prepared and distributed. Annual and semiannual reports are distributed that summarize quality control data for each analyte and for each lot of quality control material by the number of measurements per lot, the mean analytic values and standard deviations and the commercial kit or analytic method. Our reports are used by laboratories to assess their individual performance and by manufacturers to assess their method(s) performance. NSQAP also works with public and private laboratories to develop and distribute dried blood spot materials for appropriate use with new testing technologies and deoxyribonucleic acid (DNA)-based methods.

Quality assurance for filter paper

The performance of filter paper blood collection products is monitored by NSQAP. Before a new lot of paper entering the field, NSQAP evaluates the materials to verify the manufacturer's own testing process. This ensures that new filter paper lots are consistent with established guidelines and that they perform similarly to previous lots of paper.

A filter paper disk punched from a blood-filled circle provides a volumetric measurement that is similar to liquid measuring devices. Punches can be eluted in test tubes or microwell plates with accurate volumes of an elution buffer or other appropriate reagent. Eluates are aliquoted for assay by quantitative transfer using manually calibrated pipettes or by a calibrated automated pipetting system.

Isotopic enrichment of whole blood. To ensure the accuracy of the filter paper device, an isotopic method was developed by NSQAP to assess the uniformity and absorption characteristics of the paper within and between production lots as described in a NCCLS publication (Hannon et al. 1997). The NCCLS standard gives the limits for the printed circle size of the filter paper collection device. These limits provide a gauge for the volume of blood applied to a blood spot in routine collections to standardize the volume within a device and between devices.

Briefly, the method that NSQAP routinely uses begins with red blood cells that have been washed three times with saline to remove plasma and buffy coat, which contains the fibrin for clotting. This homogenizes the blood to provide a similar matrix each time whole blood pools are prepared for application to filter paper. The washed red blood cells are mixed with plasma or serum to give an adjusted hematocrit of 55% ± 1%. The whole blood is enriched with ^{125}I-L-thyroxine (~30 μCi, 11.1 × $10^8$$\beta$q), lysed by one freeze thaw cycle, mixed by constant gentle stirring and applied to a selected sampling of production lots of Food and Drug Administration-approved filter paper in 100-μl aliquots. The blood is added to the center of the printed circle and allowed to diffuse out. The blood spots are air dried overnight at ambient temperature. Punches of 3.2 mm (one-eighth-inch disks) were taken from the center, and four peripheral locations (north, south, east and west). The blood spot punches and appropriate total count tubes are counted in a gamma-counter. The absorption time and diameter of the blood spots are measured, and the blood volume for 3.2-mm disks is determined by equating the mean value of the gamma-counts per minute of the 3.2-mm disks with the counts per minute for the total counts per unit volume of liquid blood (total counts tube). A hierarchical, nested analysis of variance is used to assess the homogeneity of filter paper lots. If lot means are significantly different (statistically) from one another, decisions have to be made about whether to accept the filter paper lot for distribution (Hannon et al. 1997). The standardization of the filter paper blood collection device and subsequent analytical measurements has lead to the development of accuracy-based amino acid reference materials in dried blood spots (Chace et al. 1999).

Using the method described here, three parameters of filter paper performance were examined by NSQAP: chromatographic effects, hematocrit and blood spot volume. These parameters may affect the volume of the sample aliquot taken from a punch within the dried blood spot. These effects are important to control when making in-house control materials, proficiency-testing materials or when considering the application of blood spot-testing technology to adolescent or adult testing schemes.

Chromatographic effects. Chromatographic effects were investigated to determine whether the fibers in the filter paper matrix influence the spread of blood across the spot when the blood was applied in a single application to the center of the printed circle (Adam et al. 2000). Data from the past 3 y of evaluation studies were statistically assessed for different lots of quality control materials. Within a manufactured lot of filter paper, the average serum volume of the peripheral punches (north, east, south and west positions) from within the circle was compared with the average of the center punch. **Table 1** shows the average spot volume for the center punch (3.2-mm punch size) compared with the peripheral punches. The data indicated that the center punch had a slightly higher volume than did the outside punches. The volumes differed by 1–2% for each filter paper lot, thus, demonstrating that chromatographic effects account for <2% of the overall variation, as long as the punched disks stayed within the confines of the recommended printed circle size.

Hematocrit effects. The effect of hematocrit on blood volume for 6.0 mm (one-fourth-inch) punches was investigated (Adam et al. 2000). Five whole blood pools with hematocrit levels ranging from 30 to 70% were prepared. Each pool was spiked with ^{125}I-L-thyroxine and the blood was dispensed onto printed circles in 100-μl spot volumes of filter paper lots supplied by two manufacturers of blood collection papers. After the blood air-dried overnight at ambient temperature, a 6.0-mm punch was taken from the center of each spot. The larger 6.0-mm punch size was chosen to ensure that an adequate surface area of the filter paper was studied. Fifty replicate spots were punched from each hematocrit level and from both lots of filter paper. We compared the mean volume from a selected quality control lot (55% hematocrit) with the mean volume for the different hemat-

TABLE 1

Chromatographic effect on punch volume, where the center punch (3.2-mm, one-eighth-inch) was compared with peripheral punches taken from 100-μl volume dried blood spots of 55% hematocrit blood

Lot number	Peripheral punch average[1]	*n*	Center punch average	*n*	% difference[2]
Manufacturer 1 lot 2	1.445 ± 0.114	500	1.474 ± 0.123	125	1.97
Manufacturer 1 lot 3	1.448 ± 0.104	700	1.465 ± 0.104	175	1.16
Manufacturer 1 lot 4	1.459 ± 0.242	300	1.487 ± 0.226	75	1.88
Manufacturer 2 lot 1	1.462 ± 0.066	700	1.476 ± 0.073	175	0.95
Average % difference					1.49

[1] Serum volume (μL) in 3.2-mm punches representing north, south, east and west of the center punch.
[2] Percent difference calculated from the center punch to the peripheral punches.

ocrit levels on the two lots of filter paper. **Figure 1** shows the serum volumes of the different hematocrit levels for both lots of filter paper. A 6.0-mm punch from the center of a 100-μL spot made with 30% hematocrit blood contained ~47% more serum volume than a punch taken from a spot made using 70% hematocrit blood.

Effect of blood spot volume. The relationship between the size of a blood spot (volume of whole blood per spot) and the volume of blood in a 6.0-mm punch taken from the center of the blood spot was investigated. Whole blood spiked with ^{125}I-L-thyroxine was prepared as described above. Five different volumes of blood ranging from 25 to 125 μl were applied to one lot of each filter paper supplied by the two manufacturers. The blood was allowed to air-dry overnight. A single 6.0-mm punch was taken from the center of 50 blood spots, from each volume spotted and for each lot of paper. The radioactivity of the center punches (gamma-counts per minute) was compared with the gamma-counts per minute in a fixed volume of liquid blood. These measurements were used to determine the serum volume of the center punches. **Figure 2** shows a positive slope in serum volume per 6.0-mm punch as the blood spot volume increased. We observed a 13% increase (approximate) in serum volume from the lowest blood spot volume (25 μl) to the highest (125 μl). Good agreement between papers from the two sources was also observed.

These data demonstrated the importance of controlling such parameters as blood spot volume and hematocrit when preparing dried blood spot materials to be used as in-house quality control materials, calibration materials, reference materials or materials to be distributed for analysis in proficiency testing. NSQAP primarily prepares such materials for newborn testing. However, dried blood spots have numerous research, epidemiological and diagnostic uses that can be applied to older children and adults. It is important to bear in mind the age of the target population and to develop appropriate dried blood spot quality control and calibration materials with a hematocrit representative of that population.

Dried blood spot specimen collection, transport and storage

Collection. Blood is most easily collected from heel or finger punctures by using single-use lancing devices, many of which have been engineered to cut at precise incision depths to minimize pain and bruising (Turner and Holman 1978, Blumenfeld et al. 1979). Although somewhat invasive, this method of collecting blood is a widely accepted method for obtaining blood specimens from infants and young children, and it provides a minimally invasive way to collect blood from adolescents and adults. The NCCLS standard (Hannon et al. 1997) describes the procedures for the collection of blood from heel sticks that are summarized below. These procedures can also be generally applied to collecting blood spot specimens from finger sticks.

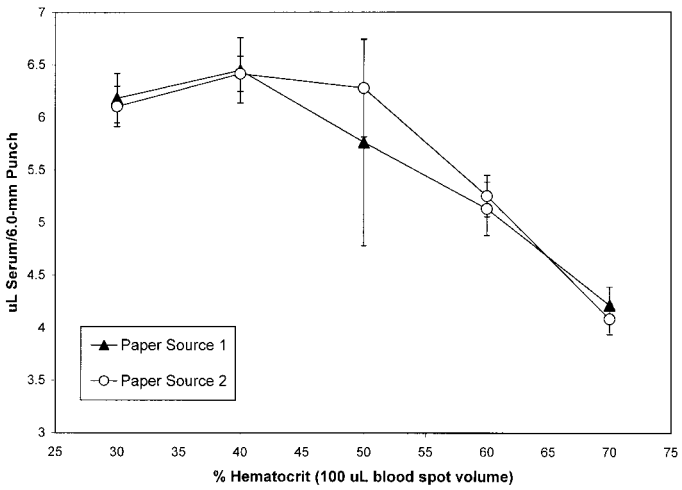

FIGURE 1 The effect of hematocrit (%) on the volume of serum (μl) in 6.0-mm (one-fourth-inch) punches. As the hemaocrit of the spotted blood increased, the amount of serum in the punch decreased. For each point, $n = 50$.

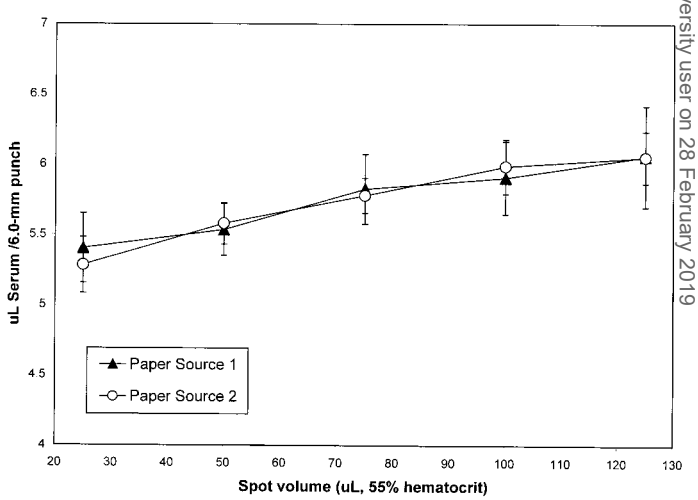

FIGURE 2 As the volume (μl) of whole blood applied to each spot increased, the amount of serum in a 6.0-mm center punch also increased. For each point, $n = 50$.

Instructions for specimen collection

- Complete each item on the newborn screening collection form.
- Do not touch any part of the filter paper circle before or after collection.
- Select puncture site and cleanse with 70% of isopropanol.
- Use a sterile, disposable lancet with 2.0-mm point or less.
- Keep the infant's heel in a down position at or below the heart level. (Sampling after a feeding promotes better blood flow.)
- Wipe away the first blood of drop.
- Use the second large blood drop to apply to the surface of the filter paper circle.
- If not completely filled, add a second large drop immediately. Fill one circle at a time.
- Fill circles from only one side of the filter paper. Fill all required circles completely.
- Dry specimen at ambient temperature for 3–4 h in horizontal position.
- Forward specimen to the state newborn-screening laboratory within 24 h.
- Improperly collected samples will be rejected by the laboratory, require a second specimen and force a delay in the analysis, thus delaying treatment of affected newborns.

Dried blood spot specimen drying and storage. It is very important to dry blood spot specimens completely before storage or transport, because moisture may harm the specimen by inducing bacterial growth or altering the elution time of the specimen. Blood spot specimens should be dried for at least 3 h over an open nonabsorbent surface at 15–22°C. The paper should be kept away from direct sunlight and should not be heated, stacked or allowed to touch other surfaces during the drying process. The filter paper containing the dried blood specimen should be protected by a sturdy paper overlay such as glassine paper (weighing paper). The specimens should be protected against humidity and moisture by packing them in low gas-permeable zip-closure bags with desiccant packages and humidity indicator cards (Hannon et al. 1997). Dried blood spot specimens protected in this manner can be stored at −20°C for many weeks or years (Behets et al. 1992, Therrell et al. 1996, Chace et al. 1999). Human immunodeficiency virus (HIV) antibodies have been shown to be stable for at least 6 mo when stored with desiccant at −20 or 4°C (Hannon et al. 1989).

Transportation of dried blood spot specimens. Dried blood spot specimens that have been packed as described above (covered with a paper overlay and stored with desiccant in a zip-closure bag) can be transported through the mail. The zip-closure bag containing the packed specimens should be enclosed and sealed in a high quality bond envelope for shipment (Knudsen et al. 1993). Packaging dried blood spot specimens in multiple layers protects mail handlers from accidental exposure and ensures specimen integrity during shipping.

Using dried blood spot specimens for research

The combination of easily puncturing the skin for the collection of blood onto paper has become a significant tool for screening individuals for clinical purposes and for carrying out epidemiological studies for the detection of numerous biological markers. Such studies have relevance for the advancement of analytical method development and public health knowledge (Therrell et al. 1996). Studies that have used dried blood spot specimens include the surveillance of HIV infection among childbearing women (Gwinn et al. 1991), serodiagnosis of infection (Parker and Cubitt 1999, Coates et al. 1998, Parker et al. 1999) and markers for autoimmune disease and coagulation factors (Nelson et al. 1998). The screening for therapeutic drugs (Patchen et al. 1983, Mei et al. 1998) and for drugs of abuse (Henderson et al. 1997) has also used dried blood spot specimens. **Table 2** provides a partial list of the numerous analytes cited in the literature that have been measured from dried blood spot specimens for epidemiological studies. This list covers an impressive range of molecular weights, from small molecules such as amino acids to macromolecules such as enzymes and DNA. In general, any analyte that can be measured from whole blood, serum or plasma can be measured from dried blood on filter paper. The dried blood matrix stabilizes many analytes, including DNA, which allows one to measure both phenotype (biochemical marker) and genotype (mutation or polymorphism) from one specimen (McCabe 1991), or to conduct multiplex DNA amplification for the detection of multiple gene mutations (Caggana et al. 1998). Parker and Cubitt (1999) provide a review of molecular methods for the detection of HIV nucleic acids.

Ethical issues. The advances in clinical testing and molecular technologies have created new opportunities for research using dried blood spots. Several issues have arisen concerning the banking and ethical use of leftover newborn dried blood spot specimens (Therrell et al. 1996, American Academy of Pediatrics, Newborn Screening Task Force 2000). These include the cost, infrastructure and procedures for storing and retrieving residual specimens; the ownership of the specimen and the informed consent process; the review process for research proposals asking to use stored specimens; written policies for the retrieval of specimens; and the clearance process for review by institutional human subjects committees that ensure patient confidentiality. Individual states should establish appropriate storage conditions, uses and consumer protections for their stored newborn dried blood spot specimens (American Academy of Pediatrics, Newborn Screening Task Force 2000). Policies for the release, use and the length of storage time for residual blood spots specimens vary by state (Hannon et al. 2000). Any group wishing to use dried blood spot specimens for research must develop guidelines for patient consent and human subjects review.

The use of filter paper for the collection and analysis of whole blood specimens has been shown to be an important way to screen newborn specimens for metabolic and heritable disorders, to further epidemiological research of public health importance and to develop analytic methods of clinical relevance. Yearly assessments of filter paper lots to ensure the uniformity of the paper over time are conducted by the NSQAP of the CDC. This interaction with industry imparts a level of confidence in the product that is then translated into devices used by states for the collection of blood from newborn heel sticks. This has lead to the broad applications of dried blood spots in other scientific investigations. The quality of the filter paper product for dried blood spot specimens has been assessed through studies of the effects of chromatography, volume and hematocrit. It was shown that factors that can contribute variation to the analytic testing process, such as hematocrit and blood spot fill volume, should be minimized. Chromatographic effects accounted for $< 2\%$ of the overall variation, whereas keeping blood volume constant while varying the hematocrit resulted in 47% less serum volume for 6.0-mm punches taken from spots made with 30% of hematocrit blood compared with spots made using 70% of hematocrit blood. Keeping the hematocrit constant while varying the blood volume of spots resulted in a 13% difference in serum

TABLE 2

Analytes measured from human blood collected and dried on filter paper

Acarboxyprothrombin
Acylcarnitine
Adenine phosphoribosyl transferase
Adenosine deaminase
Albumin
α-Fetoprotein
Amino Acids profiles
  Arginine (Krebs cycle)
  Histidine/urocanic acid
  Homocysteine
  Phenylalanine/tyrosine
  Tryptophan
Andrenostenedion
Antipyrine
Arabinitol enantiomers
Arginase
Benzoylecgonine (cocaine)
Biotinidase
Biopterin
C-reactive protein
Carnitine
Carnosinase
CD4
Ceruloplasmin
Chenodeoxycholic acid
Chloroquine
Cholesterol
Cholinesterase chemokines
Conjugated 1-β hydroxycholic acid
Cortisol
Creatine kinase
Creatine kinase MM isoenzyme
Cyclosporin A cytokines
D-penicillamine
De-ethylchloroquine
Dehydroepiandrosterone sulfate
DNA (polymerase chain reaction)
    acetylator polymorphism
  Alcohol dehydrogenase
  α 1-Antitrypsin
  Cystic fibrosis
  Duchenne/Becker
  Muscular dystrophy
  Glucose-6-phosphate dehydrogenase
  Hemoglobinopathies
    A,S,C,E
    D-Punjab
  β-Thalassemia
  Hepatitis B virus
  HCMV
  HIV-1
  HTLV-1
  Leber hereditory optic neuropathy
  MCAD
  mRNA
  PKU
  Plasmodium vivax
  Sexual differentiation
21-deoxycortisol
Desbutylhalofantrine
Dihydropteridine reductase
Diptheria/tetanus antitoxin
Erythrocyte arginase
Erythrocyte protoporphyrin
Esterase D
Fatty acids/acylglycines
Free β-human chorionic gonadotropin
Free erythrocyte porphyrin
Free thyroxine (FT4)
Free tri-iodothyronine (FT3)
Fumarylacetoacetase
Galactose/gal-1-phosphate
Galactose-1-phosphate uridyl transferase
Gentamicin
Glucose
Glucose-6-phosphate dehydrogenase
Glutathione
Glutathione perioxidase
Glycocholic acid
Glycosylated hemoglobin
Halofantrine
Hemoglobin variants
Hexosaminidase A
Human erythrocyte carbonic anhydrase I
17-α Hydroxyprogesterone
Hypoxanthine phosphoribosyl transferase
Immunoreactive trypsin
Lactate
Lead
Lipoproteins
  (a)
  B/A-1
  β
Lysozyme
Mefloquine
Netilmicin
Phenobarbitone
Phenytoin
Phytanic/pristanic acid
Progesterone
Prolactin
Prolidase
Purine nucleoside phosphorylase
Quinine
Reverse tri-iodothyronine (rT3)
Selenium
Serum pancreatic lipase
Sissomicin
Somatomedin C
Specific antibodies
  Adenovirus
  Antinuclear antibody
  Arbovirus
  Aujeszky's disease virus
  Dengue virus
  Dracunculus medinensis
  Echinococcus granulosus
  Entamoeba histolytica enterovirus
  Giardia duodenalisa
  Helicobacter pylori
  Hepatitis B virus
  Herpes virus
  HIV-1
  IgE (atopic disease)
  Influenza virus
  Interlukins
  Leishmania donovani
  Leptospira
  Measles/mumps/rubella
  Mycobacterium leprae
  Mycoplasma pneumoniae
  Onchocerca volvulus
  Parainfluenza virus
  Plasmodium falciparum
  Poliovirus
  Pseudomonas aeruginosa
  Respiratory syncytial virus
  Rickettsia (scrub typhus)
  Schistosoma mansoni
  Toxoplasma gondii
  Trepenoma pallidium
  Trypanosoma cruzi/rangeli
  Vesicular stomatis virus
  Wuchereria bancrofti
  Yellow fever virus
Specific antigens
  Hepatitis B virus
  HIV-1
Succinylacetone
Sulfadoxine
Theophylline
Thyrotropin (TSH)
Throxine (T4)
Thyroxine-binding globulin
Trace elements
Transferrin
Transferrin receptor
Uridine diphosphate-galactose-4-epimerase
Urea
Uroporphyrinogen I synthase
Vitamin A
White blood cells
Zinc protoporphyrin

volume from 6.0-mm central punches taken from the lowest spot volume compared with the highest spot volume.

The intensive study of the filter paper matrix has given NSQAP the unique expertise to provide analyte-specific dried blood quality control materials and proficiency-testing materials to laboratories screening newborn specimens. NSQAP plays an important role in maintaining the quality of newborn-screening testing worldwide. In addition, the services and technical assistance delivered by NSQAP provide a resource for those wishing to apply new analytic methods to the filter paper matrix for the advancement of clinical testing and for research endeavors of public health relevance.

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