PART I

HISTORY, APPLICATIONS, AND HEALTHCARE

1

OVERVIEW OF THE HISTORY AND APPLICATIONS OF DRIED BLOOD SAMPLES

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1.1 HISTORY

"Everything should be made as simple as possible, but not simpler."

Albert Einstein (1879–1955)

For almost 100 years, the simple concept of applying biological fluids to filter paper, drying, transporting to a laboratory, and taking aliquots has been adapted into a variety of innovative methods to generate a suitable specimen and sampling matrix for analytical testing. These methods for collecting blood and other biological fluids created a novel way to sample biological fluids and are responsible for initiating the first interest in analytical micromethods (Schmidt, 1986).

Filter paper sampling greatly simplified blood sample collection, handling, and storage over other methods in use at the time. Over the years, the availability of analytically acceptable dried blood spots (DBSs) has significantly impacted a variety of fields of study including newborn screening (NBS); epidemiology (field testing); infectious diseases; environmental research; forensics; therapeutic drug monitoring; illicit drug analysis; toxicology; and toxico- and pharmacokinetic studies of drugs and candidate drugs. Filter paper samples, although conceptually simple with few anticipated matrix effects, have resulted in many unexpected analytical complexities. The utility and acceptance of DBSs have increased over the last 30 years primarily because of concerted efforts to control, minimize, and eliminate these analytical variations.

Perhaps the earliest reference to blood collected on paper can be seen in the well-preserved Mayan murals of Bonampak, Mexico, dated 780AD. One part of the mural shows women pricking their tongues, fingers, and lips while letting the blood drops collect on paper that is then placed in a container and burned to summon the gods. Another section of the illustration portrays a child spreading his fingers with one finger being pricked for blood collection on paper. A cup on one end on the table appears to contain smoldering blood spots. A row of blood-spot images is depicted on the table's edge (Miller, 1995).

With his modification of the Wassermann test in 1911, Noguchi reported using hemolytic amboceptor (old term for hemolysin/antibody) serum absorbed onto filter paper to increase the stability of this reagent for his complement fixation method for syphilis (Noguchi, 1911). But the first credit for using filter paper for specimen collection is attributed to Bang, who reported its use in this manner for an analytical method in 1913 (Bang, 1913). His ingenious approach introduced a method for absorbing blood onto filter paper, drying the spots, and then determining glucose concentrations from the eluate. He first mentioned his specimen collection method in 1907, although it was not published until 1913 (Schmidt, 1986). His method for blood sugar analysis was both practical and reliable. Bang introduced the use of micro-samples of blood (about 100 mg) absorbed into prewashed and dried filter paper and weighed on a balance to measure the sample aliquot size. This sampling and testing technique was designated "Bang's Method" (Van Slyke, 1957; Schmidt, 1986). Bang also performed Kjeldahl nitrogen/protein determinations with a filter-paper specimen-based micromethod. Ivar Christian Bang has been declared the "founder of modern clinical microchemistry" (Schmidt, 1986).

In 1924, Chapman reported using several varieties of absorptive papers in his studies and noted that "any good

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grade of a nonalkaline filter paper of low ash content and good absorption power may be used" (Chapman, 1924). He typically used Schleicher and Schuell No. 595 paper but reported that "Whatman No. 3 paper was found to be most satisfactory for the Wassermann test." Chapman conducted a series of investigations to determine whether patient's blood could be collected on filter paper, dried, and then used in the complement fixation test for syphilis. He used DBSs for the following reasons: (1) less blood was necessary (especially important for children); (2) blood collection supplies were simple and inexpensive; (3) risks of specimen spoilage (by bacterial contamination) or hemolysis could likely be eliminated; and (4) specimen preservation for long periods of time with little deterioration would be possible. Blood was absorbed onto filter paper strips (about 4 in.), dried, and then cut into smaller pieces for elution and analysis. Chapman also noted that blood collected on filter paper and dried could be kept for at least 1 month with little deterioration of its complement fixing power (Chapman, 1924).

By 1939, filter paper was more commonly used as a transport medium for blood samples for serological examination (Zimmermann, 1939). Different types of filter paper were available and recommended or preferred by different investigators. For example, one investigator declared that only Canson 435 blotter paper was usable for his studies, while electrophoresis paper and membrane filters were not suitable (Vaisman et al., 1963). Interestingly, serum collected on filter paper was thought to be less satisfactory than blood. Additionally, efficient extraction techniques were considered to be problematic, so elution methods were recommended. Zimmerman evaluated different blotter and filter papers to determine the best product for his test. These papers included Canson 435 blotting paper and several filter papers: Delta No. 310, Schleicher and Schuell No. 589, and Whatman No. 1 (Zimmermann, 1939). He used a sharp hollow pipe to punch 15 mm paper circles for sampling. The amount of serum and whole blood in a sample was determined by weighing. Samples were declared dry after 2 hours of air drying at ambient temperature. Zimmerman also investigated the impact of different solutions, temperatures, and elution times on DBS elution efficiency (Zimmermann, 1939). The best elution was reported to be obtained from phosphate-buffered saline at pH 7.2 when eluted for 2 hours at 37°C. All papers studied were suitable for transporting dried blood and serum. Consequently, the paper with the smallest variation in the absorbed quantity of blood and serum (determined by weighing dried paper punches before and after soaking in blood or serum) was preferred (Zimmermann, 1939).

In 1950, Hogan noted that "A simpler method of collecting blood would be useful for diagnostic activities in general and would be particularly desirable for the diagnosis and treatment of congenital syphilis" (Hogan, 1950). "Indeed the nonavailability of such a simple test has hampered congenital syphilis control programs" (Hogan, 1950). He described a syphilis test that used whole blood from a finger prick collected on filter paper and dried (Eaton and Dikeman No. 613). Strips of paper were saturated with blood from a finger stick (heel, toe, or ear lobe puncture in infants and children) and the strips were air dried on a clean flat surface. Small squares were cut from the dried filter paper strips and microscopically analyzed. This procedure avoided jugular punctures in infants and children—the standard blood specimen collection procedure at the time. Although the new DBS test was not considered as "good" (sensitive) as the standard liquid serum serologic test, it fulfilled an apparent need (Hogan, 1950). The filter paper method was particularly desirable when screening children for congenital syphilis, especially for mass testing programs and home collections. The finger prick procedure quickly overcame parents' objections to jugular punctures on infants and small children. Nevertheless, some investigators continued to downplay the use of DBSs because of the lower test sensitivity when compared with liquid samples in standard laboratory tests and suggested using these DBSs only when no other specimen collection method was possible (Freeble and Orsburn, 1952).

Filter paper techniques for collecting whole blood and serum for studies on eastern equine encephalomyelitis were reported in 1957. However, the reporting investigators found that hemolytic eluate from the dried blood samples impeded readings on samples with low titers for complement fixation reactions (Karstad et al., 1957). They determined that paper discs made of highly absorbent, commercially available white paper were equivalent to discs cut from white blotting paper. Schleicher and Schuell filter paper was reported to be superior in uniformity and tensile strength when wet. Blood samples were collected from birds and horses, and 12.5 mm discs of the type used for antibiotic sensitivity tests were used. Serum was dropped onto the discs until they appeared saturated. Discs of 12.5 mm and 15 mm diameter were found to absorb 0.16 mL and 0.20 mL serum, respectively, and overnight elution was reportedly most effective (Karstad et al., 1957). Adams and Hanson found that exposure of paper discs to 56°C for 1 hour or exposure to 37°C for 7 days resulted in no appreciable titer loss for neutralizing antibodies; and therefore, the technique was declared valuable for epizootiological studies (Adams and Hanson, 1956).

Early methods to obtain DBS aliquots relied on scissors, copper tubes, or precut paper discs. In 1957, the first use of a paper-hole punch device for collecting aliquots was reported. "The filter paper strip is dipped into the blood and allowed to saturate half of the filter paper strip, dried at ambient temperature and then one-quarter inch punches are taken from the dried-blood tip of the paper strip with an ordinary ticket hole puncher to produce standardized dried-blood aliquots for testing" (Pellegrino and Brener, 1958).

Advantages of the filter paper method for specimen collection were summarized by Wolff in 1958 (Wolff, 1958).

In particular, he noted several advantages: transmittal speed (air mail was quick and economical); minimal chance of contamination; and elimination of breakage. He also noted that in complement fixation tests, it was preferable to dry serum on blotting paper rather than to collect whole blood (Wolff, 1958). The next year, Farrel and Reid (1959) conducted assays for poliovirus antibody using paper discs moistened with serum and applied to the surface of poliovirus-injected agar (with overlaid tissue culture to show zones of inhibition for the poliovirus).

Also in 1958, a method for collection of urine from infants onto squares of Whatman filter paper was described as part of a program for early detection of phenylketonuria (PKU) (Berry et al., 1958). The method involved placing a 2 in. square absorbent filter paper into an infant's diaper, allowing it to become wet (or dipping it in urine), and then allowing it to dry. If the urine appeared very pale and dilute, then the square was dipped a second time and dried again. Lead pencil identification marks on the filter paper did not interfere with any of the tests described. A 1×0.5 in. rectangle was cut from the filter paper that contained approximately 50 μL urine (Berry et al., 1958). This technique of using dried urine papers was proven important in testing for phenylpyruvic acid and a number of other excreted substances caused by metabolic defects (Berry et al., 1958; Berry, 1959).

A figure published as part of a January 1961 report (Anderson et al., 1961) was the first to illustrate inked rings (circles) printed on the filter paper as targets for blood collection. Earlier publications had only indicated techniques for dipping paper strips into blood or saturating paper squares or discs before allowing them to dry. In the inked target procedure, small quantities of blood were to be placed inside the printed circles. For convenience, four circles were printed on a small sheet of filter paper and the volume of blood needed to fill a circle was determined to be 65 μL. For field use, sheets of filter paper were placed in booklets separated with cellulose sheets. Dried specimens were placed in envelopes and mailed to a central laboratory where batches of tests could be performed with standardized reagents (Berry et al., 1958).

In November 1961, Dr Robert Guthrie reported a unique bacterial inhibition assay (BIA) for measuring phenylalanine to detect PKU. He reported collecting specimens by absorbing a few blood drops from a heel stick into special filter paper (Guthrie, 1961). The combination of an easily transportable specimen and an inexpensive and accurate screening test made large-scale screening for PKU feasible. Subsequently, NBS for PKU using DBSs became widespread as a critical prevention strategy in public health practices. Despite the fact that various techniques using filter paper as a collection medium for biological samples had been reported for at least 50 years (several times as a newly developed technique), many investigators at the time attributed the first use of DBSs to Guthrie, often referring to them as "Guthrie spots." This trend even continues today.

For Guthrie's PKU test, blood from a skin puncture was spotted onto a piece of filter paper (Whatman No. 3), dried, and mailed to his testing laboratory where 200 specimens could be tested in a batch by a single technician. The DBSs were steamed to coagulate blood proteins, after which discs were punched with an ordinary paper-hole puncher from each spot (Guthrie and Susi, 1963). The method provided a simple way of sample collection for screening babies at the time of discharge from a birthing hospital. The first case of PKU detected by the Guthrie procedure occurred in a pilot study at the Niagara Falls City Health Department Laboratory (New York) after only 800 newborns had been screened (Guthrie and Susi, 1963). For a period of time, Guthrie's laboratory also confirmed PKU from dried urine-impregnated filter paper collected by the mother 2–3 weeks after discharge and mailed to his laboratory. This procedure was later discontinued because it lacked sufficient sensitivity (Guthrie and Susi, 1963).

Interestingly, Guthrie's 1961 article was initially rejected because another recently published study using the Guthrie BIA had experienced a high number of false-positive results and had recommended not using Guthrie's test for PKU screening (Scheel and Berry, 1962). Guthrie later noted that a different filter paper source used in that study might explain their high false-positive rate (Guthrie and Susi, 1963). He felt that the filter paper used by other investigators (Scheel and Berry, 1962) might not be absorbent enough for uniform spotting. Guthrie noted that Schleicher and Schuell Grade 903 filter paper was best suited for collecting uniform DBSs and developed some minimal criteria for uniform DBS assay performance. In particular, the DBS should appear similar on both sides of the paper, should be at least 3/8 in. in diameter (but not more than 1/2 in.), and should be located close enough to the edge of the paper to allow easy punching with a paper-hole puncher (Guthrie and Susi, 1963).

In the 1960s, Schleicher and Schuell began to manufacture a special specimen collection paper called "Grade 903" and referred to as "filter paper." This paper was highly controlled to yield a consistent cotton linter paper that contained no hardeners or additives (Harvey, n.d.). Today, blood collection papers are expected to meet the ASTM International (formerly the American Society for Testing Materials) consensus standards (Hannon et al., 2013) and are certified to meet the performance standards for sample absorption and lot-to-lot consistency as set by the approved standard, NBS-01-A6 (Blood collection on filter paper for NBS programs) of the Clinical and Laboratory Standards Institute (CLSI) (Hannon et al., 2013). The introduction of this CLSI approved standard in 1982 contributed to the quantitative consistency of filter paper within and among lots of paper produced by the manufacturer and guided the extensive applications of samples dried in this matrix across a variety of fields during

TABLE 1.1 Historical and Present Sources of Filter Paper Used for Sample Collection

Filter Paper	Year in Use[a]
Schleicher & Schuell No. 595	1924
Schleicher & Schuell No. 589	1939
Delta 310	1939
Canson 435	1939
Whatman No. 1	1939
Eaton-Dikeman (ED No. 613)	1948
Munktell No. 3	1955
Schleicher & Schuell No. 740	1956
Schleicher & Schuell Grade 903	1961
Whatman No. 3	1961
Whatman No. 4	1978
Toyo Roshi (Advantec)	1979
Schleicher & Schuell Grade 2992	1983
Macherey-Nagel	1989
Whatman BFC 180 and Grade 903[b]	2005
Ahlstrom 226[b]	2007
Munktell TPN	2009

[a]Earliest year of documented use in our literature search. The indicated year may not be the first actual usage.

[b]Bolded paper sources are approved/cleared sources by the Food and Drug Administration and are monitored routinely by the Centers for Disease Control and Prevention's Newborn Screening Quality Assurance Program for sustained adherence to established performance criteria (Centers for Disease Control and Prevention, n.d.).

the last two decades. Table 1.1 contains a list of the historical sources of "blotter" and filter paper that have been or are presently used by investigators for preparing dried biological samples.

A sample aliquot obtained from a DBS is expected to achieve the same level of accuracy and precision as that from an equal pipette volume of a liquid blood sample. Since most clinical decisions are based on measured concentrations of a biomarker, it is important that the concentration in the eluate from the extracted DBS punch replicates the level in the original blood sample. Studies have demonstrated that different lots of filter paper from sources cleared/approved by the Food and Drug Administration (FDA) for blood collection are consistent with lot-to-lot variance within established performance limits (Hannon et al., 2013). The criteria for quality filter paper are established for a specific set of parameters: (1) volume of serum contained in a 3.2 mm punch from a DBS prepared with a standard blood volume and hematocrit; (2) time required to absorb a standard size blood volume; (3) appearance of the DBS; and (4) size of the spot created by a standard blood volume. Each criterion is evaluated for new lots of manufactured paper by the Newborn Screening Quality Assurance Program (Centers for Disease Control and Prevention, n.d.) at the Centers for Disease Control and Prevention (CDC) before its release to the user community.

1.2 HISTORICAL APPLICATIONS

A 2001 table contained a list of over 175 analytes that have been measured in DBSs. The analytes varied widely and included acylcarnitines, C-reactive protein, cyclosporine A, cytokines, hepatitis B virus, gentamicin, glucose, lipoproteins, prolactin, selenium, specific antibodies for over 30 viruses and microorganisms, trace elements, vitamin A, zinc protoporphyrin, among others (Mei et al., 2001). Because of the large number of biomarkers that have been analyzed from blood and other biological specimens on filter paper, it is not feasible to describe all of the applications. Thus, a representative historical review is provided with examples selected because of their impact at the time, their overall importance, and their degree of innovation.

Early in the twentieth century, several investigators reported using blood and serum collected and dried on filter paper for serologic testing for syphilis (Chapman, 1924; Chediak, 1932; Zimmermann, 1939; Hogan, 1950). Both field and home sample collections were described. The use of blood samples collected on either blotting paper or filter paper for viral serologic studies was also reported periodically. In 1956, Adams and Hanson absorbed neutralizing antibodies of vesicular stomatitis virus on blotting paper discs (Adams and Hanson, 1956). Karstad (Karstad et al., 1957) used serum and whole blood absorbed onto filter paper discs in his studies on eastern equine encephalitis. Green and Opton in 1960 (Green and Opton, 1960) and Kalter in 1963 (Kalter, 1963) reported using whole blood on filter paper for poliovirus antibody measurements in population surveys. Anderson et al. reported filter paper blood collection for fluorescent antibody studies of schistosomes (Anderson et al., 1961). Filter paper disc methodology facilitated seroepidemiology field studies, which measured antibody titers in infants and children for many viruses including measles, mumps, poliovirus, parainfluenza virus, respiratory syncytial virus, and others (Klas-Bertil and Heimburger, 1953).

The first report of blood absorbed onto filter paper for enzyme measurements was published in 1953 (Chin et al., 1966). To simplify cholinesterase measurements, a method was developed to determine two activities from one drop of blood. This method used the same principle as "Bang's Method" (Bang, 1913), discussed earlier. With two separate blood spots, the enzymes were analyzed separately with two distinct substrates to measure two types of blood enzymes: plasma butyrylcholinesterase and erythrocyte acetylcholinesterase (Klas-Bertil and Heimburger, 1953).

In 1962, Berry considered filter paper urine samples for population-based screening programs (Berry et al., 1962). She planned to screen for conditions in which a relatively large increase in certain amino acids was expected. Two possible conditions included "Hartnup disease" and "maple sugar [sic] urine disease" in which valine, leucine, and

isoleucine were excreted in large amounts. The *Lactobacillus arabinosus* microbiological assay commonly used for these amino acids in urine was modified for filter paper urine specimens. Small pieces of filter paper saturated with urine (approximately 25 μL of urine in 0.5 in.^{2}) were dried and incubated in each of three special media inoculated with the test organism. Since the organism required valine, leucine, and isoleucine for growth, absence of one or more of these amino acids was indicated by minimal growth. Each of the special growth media lacked one of the amino acids so that no bacterial growth was observed unless the dried urine contained increased quantities of the specific amino acid (Berry et al., 1962).

The use of blood spot screening for PKU became a widespread public health program during the 1960s. The Massachusetts Department of Health decided to offer a population-based screening program for PKU in July 1962. The Guthrie BIA was used on DBSs obtained from heel sticks on newborns and screening was intended to occur in all maternity hospitals in the State. When three PKU cases were found in 4 months, participation grew rapidly and by midwinter all maternity hospitals in the state were participating. This achievement made Massachusetts the first state to have state-wide NBS (MacCready, 1963).

The first report of a unique procedure for recovering blood from DBSs and matching it with liquid serum was published in 1966 (Chin et al., 1966). Using a lancet to cut the skin and induce blood flow with forceps to hold the disc, capillary blood from a finger or heel stick was saturated onto a precut disc until no white spots were showing. Discs were then placed in small petri dishes without airtight covers to dry. Each disc was assumed to contain 10 μL of serum. Blood was eluted from the spots by loading them one at a time into a syringe barrel without a plunger and forcing 25 μL of saline through each disc. There was a good correlation between the matched liquid serum and dried blood discs. A slightly lower titer was observed from the blood discs (Adams and Hanson, 1956; Chin et al., 1966).

In 1973, NBS for congenital hypothyroidism (CH) began in Quebec, Canada, expanding the testing beyond amino acids. Dussault reported success in determining thyroxin concentrations in blood eluted from filter paper discs collected from 3- to 5-day-old newborns (Dussault and Laberge, 1973). Detecting CH within the first few days of life has been recognized by some as the single most important screening test in newborns since it allows prevention of mental retardation if lifelong treatment is initiated by 2–3 weeks of life. NBS programs began to add this testing to their ongoing PKU screening programs; and by 1978, almost one million newborns had been screened in North America. To avoid a high recall rate, a certain percentage of newborns with low thyroxin were selected for secondary screening with thyrotropin (thyroid stimulating hormone [TSH]) using the original DBS sample. The combined results of the two tests were used to better estimate the likelihood of disease (Walfish, 1976). Screening for TSH alone was used in some programs, primarily in settings where screening occurred several days after birth.

Filter paper discs have also been used to estimate lysozyme activity in tear fluid. In 1976, Mackie and Seal collected tear samples by placing a 6 mm filter paper disc in the lower conjunctival fornix and allowing it to become entirely wet (Mackie and Seal, 1976). The quantity of tears collected was determined by first weighing a bottle containing the dry 6 mm filter paper disc and then weighing it after the disc was saturated with tears. The filter paper was transferred to an agar plate for lysozyme estimation. The quantity of tears held by the disc varied up to fourfold, but the lysozyme concentration could be reported in units of activity per microliter of tears when the weight was known (Mackie and Seal, 1976).

An immunochemical test for colon cancer screening using fecal occult blood smears on specially treated filter paper was reported in 1980 (Songster et al., 1980). The test employed high titer monospecific antisera to intact human hemoglobin in a radial immunodiffusion (RID) assay. A 1/8 in. disc was punched from a uniformly dry area of the fecal-smeared filter paper using a commercial conductor's ticket hole puncher. The disc was placed on the surface of an RID plate, rehydrated, and incubated at room temperature. Perpendicular diameters of precipitin rings were measured at equivalence using indirect illumination. Punched discs soaked in standard hemoglobin hemolysate were used to validate constant sensitivity of each RID plate (Songster et al., 1980).

In 1987, McCabe first reported successfully extracting DNA from blood collected on "blotter" paper and dried (McCabe et al., 1987). His results, combined with the refinement of nucleic acid amplification techniques, made genetic testing from DBSs possible. With these advances, neonatal screening laboratories began to add genetic tests for hemoglobinopathies, Duchenne muscular dystrophy, and cystic fibrosis as second-tier tests to reduce recall rates and unnecessary confirmatory testing. However, good reproducibility from DNA extractions was technically difficult compared with routine NBS methods. While direct genotyping secondary to screening (i.e., from the same DBS) was shown to facilitate diagnostic confirmation and improve the time to diagnosis and management (McCabe et al., 1987), widespread testing as part of routine NBS protocols has developed slowly and is not yet a reality. Progress in genetic analysis with samples collected on filter paper has also been achieved using buccal, saliva, and urine samples (Harvey, n.d.).

In the late 1980s, screening for lead poisoning was widely performed by determining lead concentration in blood or the concentration of another hematological indicator such as erythrocyte protoporphyrin. While routine screening usually involved liquid blood collected in small containers, blood was also collected by finger pricks onto filter paper cards and

mailed to Guthrie's Central Laboratory for testing (Shaltout et al., 1989). Recent publications largely conclude that filter paper sampling is a viable alternative for blood-lead screening, and a few laboratories routinely perform filter paper blood-lead testing on pediatric specimens (Verebey et al., 1991; Yee et al., 1995).

Polymerase chain reaction (PCR) based methods for DBS samples have proven effective and convenient for the early diagnosis of neonatal HIV-1 infection, especially in developing countries. Samples collected and dried on filter paper have facilitated large field studies by simplifying all aspects of sample collection, storage, and transport and minimizing biohazard risks. DBSs have also been found to be satisfactory for virology studies. They have been used for monitoring the emergence of drug-resistant mutations, characterizing the genotype of transmitted virus, determining viral load, and tracking global spread (Cassol et al., 1997). Filter paper–based methods have also been used for measuring viral RNA in dried plasma spots (DPS). This DPS method is cost-effective and has broad implications for population-based research and surveillance (Hamers et al., 2009).

The introduction of tandem mass spectrometry (MS/MS) as a multi-analyte platform for NBS permitted the analysis of a wide array of analytes from a single DBS punch (Rashed et al., 1999; Chace et al., 2003). The MS/MS platform was a significant factor in introducing a recommended uniform screening panel (RUSP) for newborns using DBSs in the United States (Watson et al., 2006). The RUSP currently includes over 50 conditions (including secondary targets) using a variety of testing methods (e.g., immunoassays, high performance liquid chromatography (HPLC), isoelectrofocusing, MS/MS, enzyme assays).

The feasibility of detecting patients with several inherited lysosomal storage diseases (LSDs) using DBSs as the sample matrix in appropriate enzyme assays has been reported. Detection of LSDs is based on specific enzymatic assays using plasma, leukocytes, fibroblasts, and, more recently, DBSs. Despite the low individual incidences of these conditions, enzyme assays for their detection are being used in some NBS programs internationally and are implemented presently in a couple of US state programs, while a few other states are in start-up phases. (Civallero et al., 2006).

In 2001, Severe Combined Immunodeficiency (SCID) was identified as a possible goal for public health screening with DBS at a workshop convened by CDC in Atlanta, GA (Lindegren et al., 2004). The lack of functional T or B lymphocytes in SCID can serve as a screening tool prior to the onset of symptoms. Shortly after the workshop, Chan and Puck published a method using quantitative real-time (qRT) PCR to detect SCID in newborn by measuring in DBSs the T-cell receptor excision circles (TREC), an extrachromosomal DNA fragment uniquely created during T-cell formation (Chan and Puck, 2005). The initial results suggested that the TREC assay could be a sensitive and specific method for SCID screening. A qRT PCR TREC assay with high throughput capacity now exists (Baker et al., 2009).

The DBS samples remaining after completion of NBS (residual DBSs) are used effectively for analytical method development, method comparison, and result validation (Therrell et al., 2011). Whole blood absorbed into filter paper and dried also offers an efficient mechanism for creating a repository or bank of samples with potential for research using DNA or other stable biomarkers. DBS banking systems already exist in the US military and in a few states and countries (e.g., California, Michigan, Denmark, Thailand) (Therrell et al., 1996; Therrell et al., 2011). The use of residual DBS samples for quality assurance and public health epidemiology are generally considered important public health applications; however, their potential for research use has been controversial, primarily because of the lack of consent processes in many NBS programs. A highly successful historical example of public health research using residual DBSs was the CDC's HIV-seroprevalence survey among childbearing women that provided critical public health data on the spread of HIV (Gwinn et al., 1991).

1.3 OVERALL ADVANTAGES AND DISADVANTAGES OF DRIED-BLOOD SPOT SAMPLING

1.3.1 Advantages

DBSs can be obtained with relatively little training, require minimal manipulation at the collection site, are generally considered nonhazardous, and can be transported from remote sites easily for analysis. Filter paper collection devices are low cost, stored and handled easily, offer a stable analyte matrix, and are easily used in resource-restricted settings (Hamers et al., 2009; Harvey, n.d.). DBS samples have proven to be inexpensive and reliable for testing, particularly for large-scale testing in remote populations (Oppelaar, 1966). They offer several advantages over conventional liquid whole blood, plasma, or serum sample collection. For example, the DBS sampling method is less invasive (simple heel, finger, or ear lobe prick, rather than venous needle insertion), which helps in recruiting subjects for blood studies. Sample storage is simpler and transfers are easier because the specimen is nonbreakable and not required to be frozen or shipped on dry ice for most applications. DBSs can be conveniently collected by parents or other adults with minimal training, and they can be cheaply and easily conveyed to the testing laboratory. The collection process reduces infection risk, and the volume of blood required is smaller than the liquid blood requirements (Green and Opton, 1960; Cassol et al., 1997; Harvey, n.d.).

Over time and with experience, guidance has evolved for efficiently transporting DBS specimens. DBS samples should

not be packaged in airtight, leak-proof sealed containers (e.g., plastic or foil bags) because the lack of air exchange in the inner environment of a sealed container causes heat buildup and moisture accumulation, which causes specimen degradation. Heat, direct sunlight, humidity, and moisture are considered to be detrimental to the stability of most analytes contained in DBS samples, leading to poor analyte recoveries. Desiccant packs included in shipping containers can aid in preventing moisture accumulation; however, shipping conditions are generally not controllable, resulting in limited desiccant effectiveness (Hannon et al., 2013). DBS samples can be shipped or transported by mail or courier without expectations of occupational exposure to blood or other potentially infectious material. Nevertheless, "standard precautions" and compliance with local regulations and institutional policies are required in preparing DBS samples for shipment. Despite the fact that few pathogens are known to survive for extended periods of time in dried blood on filter paper (Hannon et al., 2013), the packaging should adhere to basic triple-packaging recommendations: (1) blood is absorbed into the paper; (2) an inner envelope or other protective cover (flap) should be present containing a biohazard label; and (3) the sample should be enclosed in an outer (gas permeable) envelope of high quality paper (or tear-resistant material) (Hannon et al., 2013).[1]

For many years, investigators have studied the relationship between temperature, humidity, and stability of biomarkers in DBSs (Cassol et al., 1997). Most analytes appear to be stable in dried matrices stored at low temperature with low humidity (∼30%), similar to a lyophilized environment (Therrell et al., 1996). An extensive search of the literature regarding stability of analytes in DBSs was of minimal value in assessing the impact of long-term storage. For stability studies, differences in markers studied, study duration, storage conditions, and data analyses made comparisons difficult. Reported studies used a variety of procedures and conditions, and most did not provide meaningful conclusions about long-term storage outcomes. Analytical reference points for assessing stability were often weak. Studies covered relatively short time periods and provided data relevant only to testing environments for identifying disorders among newborns. Interpretations of stability data were inconsistent and evaluated only analyte recoveries or concentrations for disease classifications. DBS elution studies were usually limited to fixed time intervals; therefore, data based on samples that eluted slowly could be misinterpreted as resulting from sample instability (Therrell et al., 1996).

[1]Note: The US transport standards (DMM Reference 601.10.17.8) described here are harmonized with the World Health Organization's Guidance on Regulations for the Transport of Infectious Substances (World Health Organization, 2011) and the International Civil Aviation Organization's Technical Instructions for Safe Transport of Dangerous Goods by Air (International Civil Aviation Organization, 2006).

Available data on long-term storage of DBSs suggest that, for maximum recovery of most analytes, DBSs should be stored at low temperature with controlled low humidity (Therrell et al., 1996). Stability of DBS samples has been evaluated for most analytes currently included in NBS with varied outcomes. For the majority, sample degradation is minimal when stored at room temperature for several weeks (Therrell et al., 1996). Cooler, drier conditions can extend sample stability. Hot and humid storage conditions for up to 20 weeks have been found to cause a progressive decline in HIV-1 antibody titers, especially with low titered samples. The use of gas-impermeable bags containing a desiccant improved DBS sample stability for HIV-1 antibody detection over time and is recommended for the storage of whole-blood spots on filter paper in harsh tropical field settings (Behets et al., 1992).

Several stability studies have demonstrated the extractability and stability of DNA over time in residual NBS DBSs. While genomic DNA appears to be stable under tropical conditions for at least 11 years at ambient temperature, the quality of DNA for amplifying larger DNA fragments appears to decrease in specimens stored beyond 10 years (Chaisomchit et al., 2005). A study of 70 residual NBS DBSs stored for 19 months at ambient temperature gave adequate DNA for forensic studies (Kline et al., 2002). Likewise, whole genomic amplified DNA from residual DBSs that were archived for 15–25 years were acceptable for genome-wide scans and it was a cost-effective alternative to collecting new specimens (Hollegaard et al., 2009). Conversely, the stability of non-DNA biomarkers commonly used in NBS varies by analyte and appears highly dependent on storage conditions (Strnadova et al., 2007; Therrell et al., 2011; Adam et al., 2011).

1.3.2 Disadvantages

The disadvantages and assay variables potentially affected when using filter paper samples are complex. Concerns include sample volume, humidity, hematocrit, chromatographic effects (homogeneity), analyte recovery, specimen source, anticoagulant, and filter paper characteristics. For both DBSs and other biological fluids collected on filter paper, there are reported variations in quantitative analyte recovery between dried samples and liquid whole blood or other samples. Questions regarding dried samples often concern sample homogeneity and how best to minimize this variability. Issues include the best location for obtaining the optimal specimen punch (variability across and within the specimen related to absorption and chromatographic effects), the size of sample applied to the filter paper (variations in analyte concentration dependent on sample volume), and the consistency of filter paper performance (variations in performance related to punch size). Random sampling errors related to small testing aliquots from paper

punches are also a concern. Some of these issues are discussed below.

Filter Paper Effects: Throughout the history of biological fluids collected on filter paper there have been concerns, both perceived and real, about the sources and types of paper selected for a particular analytic system. As a prime example, in an early publication Guthrie successfully used Whatman No. 3 filter paper (Guthrie, 1961). Shortly afterwards, he stressed the improvement in his test results when he used Schleicher and Schuell No. 903 filter paper, which he observed to be more absorbent (Guthrie and Susi, 1963). Commenting later on reported poor results with his method by another user (Scheel and Berry, 1962), Guthrie (Guthrie and Susi, 1963) noted that "While it is not possible to account for the discrepancy between Scheel and Berry's (Scheel and Berry, 1962) results and ours, one likely source is the filter paper used in their study, Whatman No. 3." Interestingly, Partington and Sinnott (1964) were unable to find an advantage of one filter paper over the other; and they reported that there was no evidence that the Schleicher and Schuell paper facilitated any better collection of blood from patients. Direct comparisons of BIA growth zones for the two papers using the same blood sample yielded similar results with no particular advantage to either paper (Partington and Sinnot, 1964).

While less invasive, collection of quality DBS samples requires practice and plays a major role in the quality of test results. Conversely, the quality of a liquid blood specimen is seldom identified as a factor in the quality of analytical results. As one example, when blood does not completely and evenly penetrate through the filter paper to the other side, it is generally labeled as a "poor" sample. In early comparisons with the BIA for PKU measurements, growth zones obtained from "poor" samples were the ones that were significantly smaller than those from "good" samples. A correlation factor used with poor samples assumed that they were at least half soaked, so a high correction factor was applied to guard against underestimating the phenylalanine content (Partington and Sinnott, 1964). Correction factors were eventually replaced by a request for a properly collected replacement specimen, which continues as the practice today.

As test sensitivities increased, the quality of DBS specimens became more important. Paper quality was increasingly scrutinized, particularly when radioimmunoassay techniques for detecting CH became widespread in the1970s (Dussault and Laberge, 1973). Responding to continuing complaints about filter papers' reliability and reproducibility, the CDC initiated a filter paper evaluation project in 1981 (Centers for Disease Control and Prevention, n.d.). Filter paper quality was assessed using a DBS-based quantitative isotopic test for measuring and monitoring an established set of performance characteristics. Each filter paper production lot was measured against a defined set of performance criteria. Filter paper manufacturers voluntarily collaborated in CDC's evaluation of their products as an integral part of their quality assurance protocol. This project ultimately resulted in a national standard for blood collection on filter paper developed by the CLSI, currently in its sixth edition. The standard (LA04/NBS01) defines proper specimen collection protocols and addresses related issues including paper quality (Hannon et al., 2013). The FDA defined filter paper manufactured for blood collection as a medical device and requires FDA clearance. The combination of activities above has resulted in minimizing lot-to-lot and source-to-source variations in the quality of filter paper currently used (Slazyk et al., 1988). Data from a recent CDC study (Mei et al., 2010) indicated 4–5% variability in analytical results between approved filter paper sources, and these results were similar to that observed for the lot-to-lot variability (Mei et al., 2010). It is generally agreed that for the best performance, the same filter paper lot and source are used throughout a study. A research study examining results for 28 elements (e.g., lead, iron, zinc, nickel) simultaneously measured in DBS samples produced more variation than expected. The authors concluded that random contributions from the background elements in the filter paper were the primary obstacle to measuring elements in DBSs (Langer et al., 2011).

Sample Volume: The process of collecting blood on filter paper from newborns is considered difficult by some, but result imprecision and variability can (and should) be minimized by using standardized collection procedures. Using preprinted target rings on the filter paper can help control the volume of blood collected, particularly when the target circle is filled correctly to its edge during blood collection. While not the preferred collection technique for NBS, careful blood collection using capillary tubes of predetermined fill volume may be helpful for some investigations. (Mei et al., 2001; Hannon et al., 2013). A study of the potential recovery variability from differing volumes of blood on filter paper has demonstrated the importance of controlling blood spot–volume for improved quantitative measurements. Five different volumes of blood ranging from 25 mL to 125 mL were spiked with 125I-thyroxin at a constant hematocrit and applied to paper from a single production lot from each of two filter paper manufacturers. The samples were air dried overnight and a single 6.4 mm punch was taken from the center of 50 DBSs from each blood volume applied on each filter paper lot. Isotopic count–measurements were used to compare the serum volume of the center punches. A 13% increase (approximate) in serum volume, sufficient to potentially affect analyte measurements, was observed between center punches taken from the lowest blood volume to the highest volume; however, essentially no differences were noted between the two paper sources in this study (Mei et al., 2001).

Dried whole blood, plasma, and serum may provide suitable matrices for HIV type-1 viral load determinations and for drug resistance genotyping (particularly useful in antiretroviral therapy for HIV-1-infected individuals living

in low to middle income countries). Limitations of the DBSs include reduced sensitivity resulting from small sample volume, nucleic acid degradation under extreme environmental conditions, impaired efficiency of extraction, and interferences with proviral DNA. Improved analytical sensitivity is necessary for routine applications to monitor therapeutic treatments, especially at the onset of treatment failure (Hamers et al., 2009).

Humidity Effects: Humidity is a factor in the way blood spreads in filter paper, how the sample dries, and analyte stability in the dried blood matrix. Each of these factors has been closely examined (Cernik and Sayers, 1971; Hannon et al., 2013; Adam et al., 2011). The separate contributions of elevated temperature and elevated humidity changes in levels of 34 analytes in DBS samples have been reported (Adam et al., 2011). Results from these accelerated degradation studies showed that degradation of 27 of the analytes was primarily caused by high humidity in the DBS storage environment; whereas, the degradation of 4 analytes was primarily caused by the 37°C storage temperature. Markers varied widely in the degree and rapidity of degradation during storage at both low and high humidity and elevated temperature (Adam et al., 2011). Other investigators found that IgG deteriorated faster in samples stored on filter paper relative to serum samples. As a result, precise evaluation of the serum dilution represented by the DBS eluate and its loss of activity relative to temperature variations and humidity were suggested (Guimaraes et al., 1985)

Hematocrit Effects: The newborn's hematocrit (proportion of red blood cells) can significantly affect analytical results. The blood volume within a given 3.2 mm disc may increase significantly when hematocrit levels fluctuate from 30% to 70% (Adam et al., 2000). Hematocrit variability and its effect on quantitative results represent significant analytical challenges in method validation (Carter, 1978). A 6.0 mm punch from the center of a 100 μL DBS made from 30% hematocrit blood contained 47% more serum volume than a similar punch from 70% hematocrit blood (Adam et al., 2000). Blood samples with low hemoglobin concentrations (and hence low hematocrits) spread more widely than those with normal and high hemoglobin concentrations. This was confirmed in a study of blood lead values in laboratory-prepared samples with low hemoglobin values. The results were found to be unreliable unless adjustments were made relative to the area over which the blood had spread (Carter, 1978).

Analysis of DBSs by MS/MS has vastly expanded the detection of inborn errors of metabolism in NBS. Despite its proven sensitivity, many issues related to DBS sample preparation remain unresolved (Holub et al., 2006). The hematocrit profoundly affects blood viscosity and may influence blood diffusion properties. Newborns show a considerable interindividual variability in hematocrits. A recent study showed that levels of most amino acids and guanidinoacetate increase significantly with increasing hematocrit, while hematocrit has a less pronounced effect on some amino acids. Total acylcarnitines correlated positively with hematocrit levels. In low hematocrit samples, levels of most amino acids and free carnitine were higher in the peripheral spot punches than in the center spot punch. Both hematocrit and position of the punch within the DBS can significantly affect analyte values (Holub et al., 2006).

Chromatographic Effects: Chromatographic effects can affect test results when fibers in the filter paper matrix influence the spread of analytes within the sample as blood is absorbed into the paper. In such cases, analyte concentrations can vary from the center of the specimen to the edges depending on the magnitude of the effect. A CDC study statistically assessed evaluation study data collected over 3 years for different lots of quality control materials (Adam et al., 2000). Within a manufactured lot of filter paper, the average serum volumes of peripheral punches (north, east, south, and west positions) were compared with the average of the corresponding center punch. Center punches were found to have slightly higher serum volumes (diluting the analyte concentration) than peripheral punches. The volumes differed by 1–2% for each filter paper lot, indicating that chromatographic effects may account for up to 2% of analytic variations, assuming the punched discs stayed within the confines of the recommended printed circle size. Metabolites may be distributed as a result of chromatographic effects within a spot; and therefore, punch location may have a significant effect on the metabolite concentration detected (Holub et al., 2006). Therefore, it is theoritically possible to miss an out-of-range analyte depending on the hematocrit and position of the punched disc.

Anticoagulants: Some filter paper specimen collection techniques, particularly the use of capillary tubes, may involve anticoagulants, which may affect assay results. As an example, an enzyme-linked affinity assay was used to quantify PCR products from whole blood, plasma, and separated mononuclear cells collected in the presence of four common anticoagulants: acid citrate dextrose, ethylenediaminetetraacetic acid (EDTA), potassium oxalate, and sodium heparin. In the case of sodium heparin, reduction of the product signal was observed after amplification of nucleic acid extraction from whole blood, washed mononuclear cells, and plasma. The inhibitory effects on gene amplification could be reversed with heparinase. The addition of as little as 0.05 U of heparin completely inhibited amplification of the HLA-DQ sequence from placental DNA. No adverse effects were detected with acid citrate dextrose and EDTA (Holodniy et al., 1991).

Trace amounts of EDTA have been shown to interfere with DBS NBS lanthanide fluorescence/time-resolved immunofluorometric assays (Holtkamp et al., 2008). EDTA may be distributed unevenly throughout the filter paper card, even throughout a single blood spot, especially when

EDTA-coated capillary devices are used. It is possible to use MS/MS to simultaneously detect the presence of EDTA while routinely analyzing DBSs for amino acids and acylcarnitines. This information can then be used to identify samples containing EDTA, which may potentially interfere in the analysis of thyroxine, thyrotropin, immunoreactive trypsinogen, and 17-hydroxyprogesterone (Holtkamp et al., 2008).

Analyte Extraction: Extraction solutions and buffers used with DBSs may limit the sensitivity of some tests (Zimmermann, 1939). Samples are usually diluted during extraction processes, and clean sample reconstitution may be difficult. The choice of extraction solvents and elution conditions may affect testing and extraction efficiency and can lead to assay problems (Kayiran et al., 2003). Elution efficiency with DBSs depends on the sample volume required and may vary by analyte. Depending on assay sensitivity, it may be difficult or impossible to extract sufficient amounts of an analyte from small volume DBSs (Kayiran et al., 2003). It is important for the eluate from a DBS to quantitatively reflect the original blood sample content. At least one study has demonstrated that different production lots of filter paper from a manufacturer consistently and accurately reflect the original sample concentrations (Mei et al., 2010).

Extracts from DBSs used to monitor recovery amounts of specific antibody activity have exhibited a spectrum of results related to drying times. Antibody activity of IgM immunoglobulin declines rapidly on storage, regardless of temperature. As an example, a study of yellow fever IgM immunoglobulin antibodies found that they were not detectable in eluates stored for 1 month, even at −20°C. In contrast, antibody activity residing in the IgG immunoglobulin was shown to be relatively stable on filter paper discs over a 4-month period, especially when stored at lower temperatures. Stability of IgA antibodies requires further study. Therefore, interpretation of serologic data from studies using DBS samples must account for the differences in storage stability of the various immunoglobulins classes (Cohen et al., 1969).

Blood Source: Questions often arise concerning potential differences between capillary specimens collected by skin puncture versus venous specimens. DBSs generally are not considered "standard" diagnostic samples and their results may not be directly comparable with those from liquid serum or plasma. Analytical decision levels established for diagnostic clinical specimens may not correlate well with DBS results, so different decision levels should be established for samples in a DBS matrix. In serologic studies of children, the correlation of titers of paired sera and DBSs was excellent with the exception that titers of the capillary DBS samples tended to be slightly lower than the matched serum samples (Chin et al., 1966). On the other hand, differences in analyte concentration have been reported between capillary and venous blood sample sources for cholesterol studies (Greenland et al., 1990). A study of various parameters in the complete blood count versus differential counts found differences depending on the type of blood sample used, venous or capillary (Kayiran et al., 2003). In term neonates capillary blood samples had higher hemoglobin concentrations, hematocrits, red blood cell counts, white blood cell counts, and lymphocyte counts than venous blood. With a higher hematocrit, the serum content is lower in the DBS punched disc. The terms "capillary blood" or "venous blood" should be used rather than the term "peripheral blood" when results are reported so that sample accuracy will be better reflected (Kayiran et al., 2003).

1.4 CONCLUSION

For nearly 100 years, the process of collecting biological fluids on filter paper has waxed and waned as a useful scientific application for laboratory testing. This sampling technique has been periodically rediscovered and considered to be "new." The dried sample matrix has presented unique challenges, but these have been overcome with perseverance and innovation. As with most scientific discoveries, the collection devices used and the techniques for using dried samples have improved and expanded over time, particularly in the past 25 years. Samples collected on filter paper have been useful for many different technologies and in a variety of fields of study. Today, there is widespread acceptance of DBSs and other dried biological fluids as valid samples useful for many different purposes. The future of this simple idea for collecting and transporting a valid biological sample seems unlimited. Experiences from using filter paper sample matrices are likely to evolve into similar uses with some of the newly developed non-cellulose products currently available. This evolution will likely provide both improved analytical precision and accuracy. In turn, skepticism about the use of dried samples should be minimized or eliminated when considering their efficacy in high quality research.

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