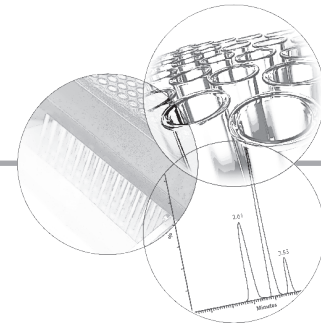

Performance properties of filter paper devices for whole blood collection

Background: The Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention assesses the adherence to established performance standards of manufactured lots of whole blood filter paper collection devices that are registered by the US FDA. We examined 26 newborn screening analytes measured from blood applied to filter papers from two FDA-cleared sources, Whatman® Grade 903 and Ahlstrom Grade 226. The dried blood spots contained analytes at both single levels and dose–response series. **Results:** We observed overlap at one standard deviation for each analyte, with no more than 4–5% difference between the papers. **Conclusion:** The data demonstrated similarities of analyte recovery between the papers, indicating comparability of the devices for newborn screening and other applications.

Robert Guthrie developed the process of collecting **whole blood** spotted onto filter paper for early detection of metabolic disorders almost 50 years ago. Guthrie also developed the first **newborn screening** (NBS) test for phenylalanine, the biochemical marker for phenylketonuria [1]. This innovation launched the field of population-based NBS using blood collected on filter paper [2]. It was noted at that time that the type and source of the filter paper used to collect blood from a newborn heel prick greatly influenced the analytical result. In 1981, these and subsequent observations about the reliability and reproducibility of the filter paper prompted the establishment of the Filter Paper Evaluation Services at the Centers for Disease Control ('and Prevention' had not been added at that time) (CDC). CDC's NBS **Quality Assurance** Program (NSQAP) helped establish the filter paper evaluation program collaboratively with the Association of Public Health Laboratories (APHL) [3]. Consequently, the Clinical and Laboratory Standards Institutes (CLSI) created and published a national benchmark for collecting blood on filter paper. This standard, CLSI LA4-A5, is now in its fifth edition and addresses issues associated with the **filter paper collection device**, collecting specimens and transferring blood onto filter paper [4]. The approved standard describes the parameters required for manufacturing filter paper that will provide homogeneous dried blood spots (DBS) of uniform size when a 100-µl aliquot of blood is applied to the paper [4]. The process of using the filter paper blood collection device, similar to other methods of collecting blood, has a level of imprecision and variability that can be characterized by standardized procedures; however, controlling the variables during the collection process in neonatal nurseries is difficult. The standard provides ways to minimize variables, such as the amount of blood spotted in preprinted circles on the paper and chromatographic effects that will influence the volume of blood found in a standard 3.2-mm punch from the spot (3.42 µl of blood) [5,6]. The newborn's hematocrit (proportion of red blood cells) effects cannot be controlled and will impact the sample aliquot, whereby higher hematocrits will have lower serum volumes in a standard punch [6]. Hematocrit will vary by patient and the existence of noncontrollable variables makes the use of this specimen matrix for quantitative measurements only a screening tool. The standard of care for NBS dictates that analytical values measured in DBS that are outside of a specified cut-off require follow-up testing to confirm the screening results. The approved CLSI guidelines provide the framework for defining how the filter paper matrix influences blood collection so that precision and reproducibility can be achieved from lot to lot. Minimizing filter paper transitions from lot to lot is essential to ensure uniformity of specimen collection, calibrators, quality control materials and other reference materials on this matrix because multiple paper lots are in use at any given time.

For almost 30 years, NSQAP has been monitoring filter paper performance using a DBS-based quantitative radioisotopic method to

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Key Terms

Whole blood: Blood containing all its components such as red and white blood cells, platelets and plasma.

Newborn screening: The public health system designed to test, detect and follow-up newborns diagnosed with treatable, inherited congenital disorders.

Quality assurance: Systematic process of activities to generate accurate and precise results.

Filter paper collection device: Pure cotton fiber (100%) paper designed to meet specific criteria for the diameter of the circle and for the absorption time of a 100-µl blood aliquot.

measure performance characteristics under a standardized protocol [4,6]. Filter paper performance is reported annually by lot and manufacturer through the NSQAP Annual Report [101]. The US FDA has registered two commercial sources of filter paper for blood collection as Class II Medical Devices (21 CFR §862.1675). The user community has requested that NSQAP staff provide an in-depth assessment of filter papers from the two commercial sources with a variety of NBS analytes. The request is based on NSQAP's long record of quality assurance services and support to public health laboratories that conduct NBS and Ahlstrom filter papers introduction as a new approved source [3]. In this study, we examined the comparative properties of the two FDA-registered filter papers by analyzing a large array of NBS analytes in blood prepared on each filter paper source; we studied Whatman® Grade 903 and Ahlstrom Grade 226 filter papers. CDC and several NBS laboratories in the USA (n = 4) and Europe (n = 2) performed the analyses.

Experimental

■ Materials & reagents

We purchased Whatman Grade 903 filter paper (Lot No. W071) from Whatman, Inc. (NJ, USA) and Ahlstrom Grade 226 filter paper (Lot Nos. 8040201 and 6460701) from ID-Biological Systems, Inc. (SC, USA). We obtained thyroid-stimulating hormone (TSH; Third International Reference Preparation, 81/565) from the National Institute of Biological Standards and Controls (Hertfordshire, UK). We purchased L-thyroxine sodium salt (T4) from Fisher Scientific (GA, USA). Sigma-Aldrich Corp. (MO, USA) provided amino acids (tyrosine, citrulline, phenylalanine, leucine and valine), 17-α-hydroxyprogesterone (17-OHP), free carnitine (C0), acetylcarnitine (C2), D-galactose and α-D-galactose-1-phosphate dipotassium salt pentahydrate type II (total galactose [TGAL]). We obtained immunoreative trypsinogen (IRT; cationic form) from Calbiochem-EMD Chemicals (NJ, USA). We purchased propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), hexanoylcarnitine (C6), octanoylcarnitine (C8), decanoylcarnitine (C10), myristoylcarnitine (C14), palmitoylcarnitine (C16) and stearoylcarnitine (C18) from Life Science Resources (WI, USA). Battelle Memorial Institute (OH, USA) provided malonylcarnitine (C3DC). We purchased hydroxylisovalerylcarnitine (C5OH) and glutarylcarnitine (C5DC) from Cambridge Isotope Laboratories (Andover, MA). All chemical reagents were used without further purification. Individual units of normal adult blood from Tennessee Blood Services Corp. (TN, USA) were used to prepare DBS with normal galactose-1-phosphate uridylyltransferase (GALT) and biotinidase (BIO) activities. We used umbilical cord blood from Cleveland Cord Blood Center (OH, USA) to assess fetal (hemoglobin F) and adult (hemoglobin A) hemoglobin proteins.

Table 1. Methods used by participants.

Analyte	Method
T4	Autodelfia† Delfia†
Thyroid-stimulating hormone	Autodelfia† Delfia†
17-α-hydroxyprogesterone	Autodelfia†
Immunoreative trypsinogen	Autodelfia† Delfia† ELISA‡
Amino acids and acylcarnitines	Derivatized-MS/MS nonkit Nonderivatized-MS/MS nonkit Derivatized-MS/MS neogram MS2 kit† Derivatized-MS/MS kit§

†PerkinElmer (Turku, Finland).
‡MP Biomedicals (OH, USA).
§Chromsystems (Munich, Germany).

■ Preparing DBS

Adult whole blood (type O) was obtained from Tennessee Blood Services Corp. and the hematocrit adjusted to 50%. We applied aliquots of 75 µl of analyte-enriched blood in tandem to blind-coded strips of Whatman and Ahlstrom filter papers, and then processed cord blood for hemoglobin analysis in the same way. Both types of filter paper were preprinted with broken-line 12-mm circles. The blood spots were dried overnight horizontally on special racks at ambient temperature. The following day, strips of DBS were separated by sheets of weighing paper (Fisher) and were placed in Bitran Series S, liquid-tight, zip-closure specimen bags (Com-Pac International, IL, USA) that contained desiccant packets (Poly Lam Products, Corp., NY, USA) and humidity indicator cards (Desiccare, Inc., CA, USA). DBS were stored at -20°C and the desiccant was changed when the indicator cards showed more than 30% humidity. We stored DBS under these conditions until the day of in-house analysis or until the specimens were shipped by express mail to reference laboratories for further analysis.

We prepared the DBS as multiple-analyte mixtures. Specimens were enriched at a high concentration so that changes associated with paper types would be measured at a precise analyte level. We prepared a dose–response (dilution) series with multiple levels of each analyte covering the analytical measurement range for analytes measured by immunoassay and MS/MS. A stock analyte mixture that contained C8 served as an internal standard to monitor linearity and accuracy of the dilution series by highly specific MS/MS methods [7]. We prepared the dilutions with split aliquots of the original nonanalyte-enriched base blood pool. We also examined TGAL, GALT, BIO and hemoglobins for performance differences among the two filter paper matrices. BIO and hemoglobin data are routinely reported qualitatively.

■ Specimen distribution, analysis & data reporting

We sent all sets of blind-coded DBS by express mail to the participating laboratories with instructions for testing and reporting data. Each laboratory assayed specimens in duplicate for two analytic runs by their routine testing methods. Each participant entered results on a provided data report form and sent them to the CDC.

Study participants included CDC and laboratories in the USA (n = 4) and Europe (n = 2). The study comprised two separate specimen distributions: the first in October 2008 and the second in December 2008. The first shipment included analytes measured by MS/MS and the second shipment covered analytes measured by immunoassays. **Table 1** identifies the variety of methods used by study participants and by analyte.

■ Analyzing serum volume in 3.2-mm standard disks (punches)

The CDC measured serum volumes according to CLSI LA4-A5, Appendix C [4]. Briefly, we enriched whole blood with hematocrit adjusted to 55% with isotopically labeled T4 (^{125}I-L-thyroxine, Perkin Elmer LAS, MA, USA). Aliquots of blood (100 µl) were pipetted onto blind-coded strips of filter paper representing a random sample of the lot. We recorded the absorption time and the diameter of each spot. After the blood spots were dried overnight at ambient temperature, we took 3.2-mm punches from the center and four peripheral locations (north, south, east and west). We counted the punches in a γ-counter and statistically analyzed data using a hierarchical, nested analysis of variance to assess the homogeneity of the filter paper lots. An F-test was used to test equivalence of the mean values (counts/min/punch) of the lots of filter paper [4].

Table 2. Whatman® filter paper lot-to-lot variance (n = 8 lots).

Year of manufacture	Lot number	Serum volume (µl)	n†	Mean serum volume (µl) n = 2675	SD	%CV
1998	W981	1.460	375	1.474	0.061	4.13
2000	W001	1.400	375			
2001	W011	1.571	375			
2003	W031	1.510	250			
2004	W041	1.440	250			
2005	W051	1.489	250			
2007	W071‡	1.397	500			
2008	W081	1.521	300			

Intact red blood cells were used to prepare the dried blood spots.
†Number of punches tested per lot evaluated.
‡Filter paper lot used in this study.
CV: Coefficient of variation; SD: Standard deviation.

Results & discussion

■ Lot-to-lot variance of filter paper

Table 2 shows the lot-to-lot variance for eight different lots of Whatman filter paper produced during approximately 10 years. Data for the Ahlstrom paper are presented in **Table 3** and show lot-to-lot variance and serum volumes similar to the Whatman paper; however, because Ahlstrom is a newly introduced source, its data encompass fewer lot numbers and a shorter time span. **Tables 2 & 3** include data for serum volume of each lot of filter paper used in this study. We examined two different Ahlstrom lot numbers in this study; however, the analyte-enriched DBS data are shown for only one lot **(Table 3)** because of the high data concordance. According to the CLSI guidelines for filter paper, the published

Table 3. Ahlstrom filter paper lot-to-lot variance (n = 6 lots).

Year of manufacture	Lot number	Serum volume (µl)	n†	Mean serum volume (µl) n = 1750	SD	%CV
2005	5431001	1.416	250	1.472	0.069	4.66
2006	6050501	1.465	500			
2007	6460701‡§	1.488	250			
2007	7181001	1.440	250			
2007	7231001	1.423	250			
2008	8040201‡	1.601	250			

Intact red blood cells were used to prepare the dried blood spots.
†Number of punches tested per lot evaluated.
‡Filter paper lot used in this study.
§Data for analyte enrichment of this filter paper lot not shown.
CV: Coefficient of variation; SD: Standard deviation.

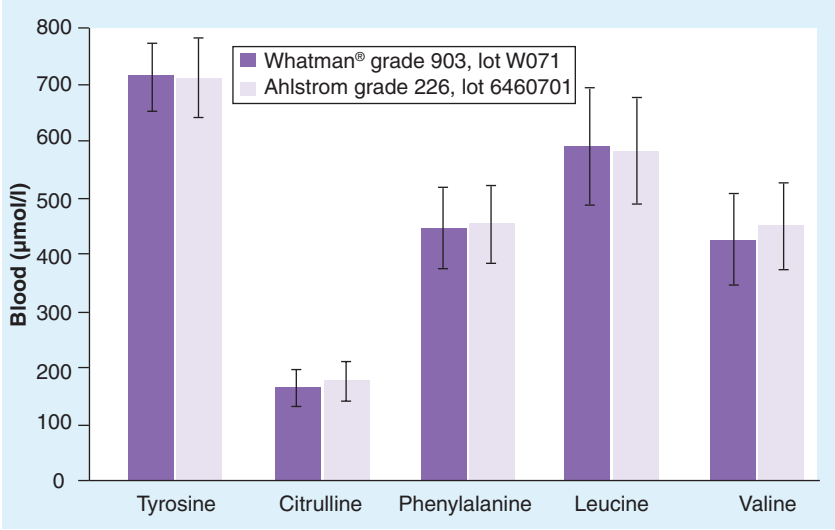

Figure 1. Recoveries of amino acids for two whole blood collection papers measured by MS/MS (n = 5 laboratories, 20 results per analyte; for valine, n = 4 laboratories, 16 results). Each bar at top of column represents the mean value ± one standard deviation.

and standardized acceptable serum volume for a 3.2-mm disk (punch) is 1.54 ± 0.17 µl (3.42 µl whole blood) for intact-cell blood spots (55% hematocrit and 100 µl spot volume) [4]. The lot-to-lot data for both the Whatman and Ahlstrom filter papers fall within the established mean volume serum per disk [101].

■ Analyzing DBS specimens

FIGURE 1 shows the recovery of amino acids from DBS specimens prepared on the two sources of filter paper. **FIGURE 2** shows the recovery of selected acylcarnitines from DBS prepared in the same way. We used a variety of MS/MS methods **(TABLE 1)** to test for amino acids and acylcarnitines. **FIGURE 3** shows the analysis of DBS specimens enriched with TGAL, IRT, T4 and TSH and prepared from normal whole blood that was also tested for GALT activity. For the hemoglobins and BIO, we observed no qualitative differences among the filter paper lots and sources. Aggregate data are shown for all figures and the error bars at the top of the measured analyte concentration columns indicate a strong overlap at one standard deviation for each analyte, regardless of the method used.

■ Analyzing DBS specimens prepared as a dose–response series

We prepared a dose–response series for T_4, TSH, 17-OHP and IRT, and selected the representative data for 17-OHP and C8 for presentation **(FIGURES 4 & 5)**. In this series, we examined the linearity of response between the paper sources by analyte. Five laboratories reported data by immunoassay methods for 17-OHP; six laboratories reported data by MS/MS methods for C8. Regardless of the type of

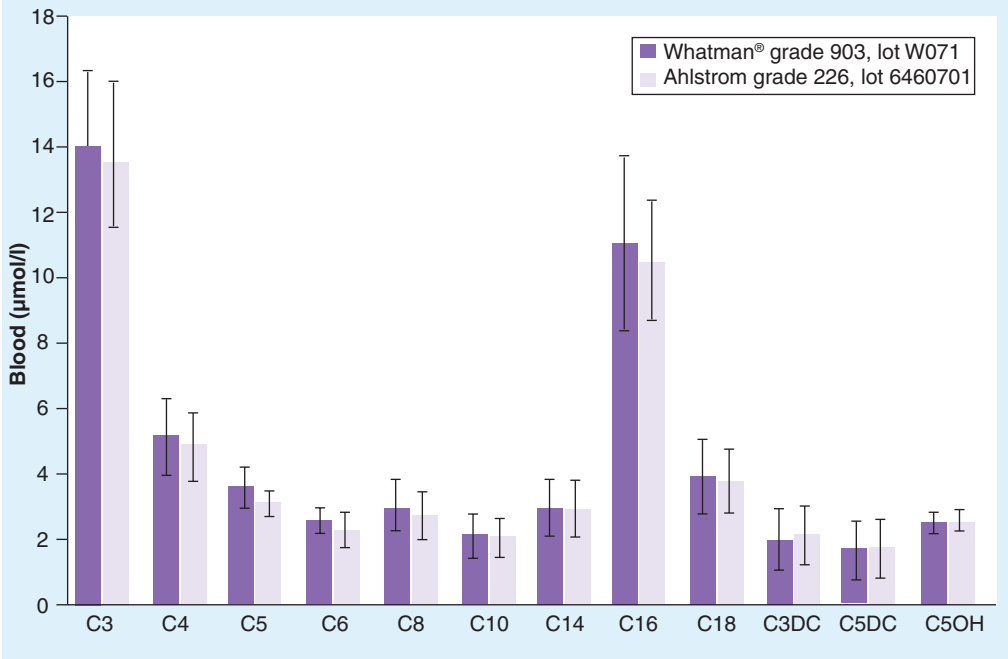

Figure 2. Recoveries of acylcarnitines for two whole blood collection papers measured by MS/MS (n = 5 laboratories, 20 results per analyte; for C10 and C18, n = 4 laboratories, 16 results per analyte). Each bar at top of the column represents the mean value ± one standard deviation.

analyte or method used, both filter papers produced linear dose–response curves that were parallel with marginal curve displacements/bias. Error bars, representing one standard deviation, show overlap for each point in the series **(FIGURES 4 & 5)**. Differences in analytical results among laboratories were less than 5%, which is similar to the serum volume difference **(TABLES 2 & 3)** noted among lots for each manufacturer (within a filter paper source).

Conclusion

We conducted an extensive investigation to assess the equivalence of two filter papers from FDA-approved commercial sources. The investigation covered most analytes routinely tested on DBS for NBS by the commonly used technologies. We enlisted both domestic and foreign laboratories to measure the multiple analyte-enriched blood spots that were identically spotted and dried on two sources of filter paper devices so that data from several laboratories and a variety of methods could be used to assess filter paper comparability. Only a selected portion of the collected study data is presented in this paper. All of the collected data are available for review on the NSQAP website [101]. The study data indicated that the difference in analytical results between manufacturers could be within at least 4–5% comparability or, at a minimum, equal to the lot-to-lot variance of a single manufacturer's filter paper products **(TABLES 2 & 3)**. This magnitude of difference was the largest observed regardless of analyte tested or method used. As discussed by Mei, hematocrit will influence the volume of serum present in a punch [6]. Patient hematocrit is variable and cannot be controlled, therefore, analytical measurements performed with DBS for NBS are preliminary and require follow-up testing. The data collected here support the conclusion that the performance of filter paper Grades 903 and 226 from two FDA-cleared/approved sources is equivalent, indicating that adherence to standard guidelines produces whole blood-collection devices that meet defined criteria and yield consistent performance.

The success of NBS and its continued expansion could not have been achieved without the co-operation of filter paper manufacturers and the user community. The CLSI standard for overseeing whole blood collection on filter paper has been practiced since 1982. NSQAP staff will continue to voluntarily monitor the performance of new production lots of filter paper from all FDA cleared/approved commercial sources as they are manufactured and before their release to the user community for routine NBS and other applications. Those

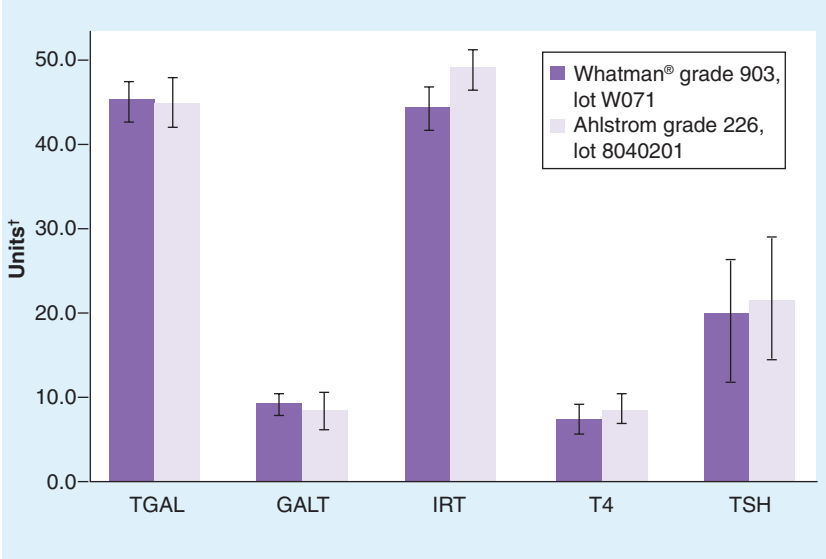

Figure 3. Recoveries of analytes measured by methods other than MS/MS. †Units for each analyte are: TGAL, mg/ml blood; GALT, U/g hemoglobin; IRT, ng/ml blood; T4, ng/ml serum; TSH, µIU/ml serum (n = 3 laboratories, 12 results for TGAL, GALT and T4; n = 5 laboratories and 20 results for IRT; n = 6 laboratories and 24 results for TSH). Each bar at top of the column represents the mean value ± one standard deviation.
GALT: Galactose-1-phosphate uridyltransferase; IRT: Immunoreative trypsinogen; T4: L-thyroxine sodium salt; TGAL: Total galactose; TSH: Thyroid-stimulating hormone.

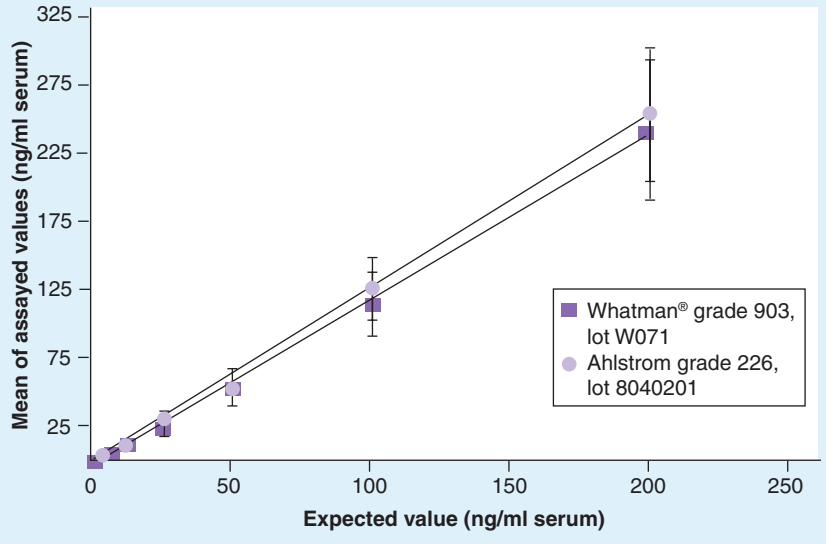

Figure 4. Recovery of 17-OHP from DBS prepared on Whatman and Ahlstrom filter papers as a dose–response series (n = 5 laboratories, 20 results per point). [0.0;1.5;3.1;6.3;12.5;25.0;50.0;100.0 ng/ml enrichments]. Each point represents the mean analytical value and each error bar represents the mean value ± one standard deviation.

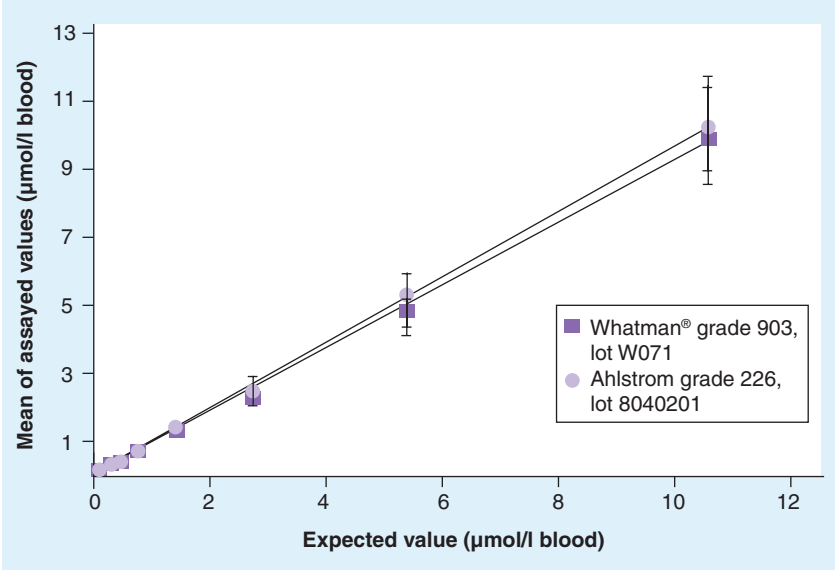

Figure 5. Recovery of C8 from dried blood spots prepared on Whatman® and Ahlstrom filter papers as a dose–response series. n = 6 laboratories, 24 results per point. 0.00; 0.15; 0.30; 0.60; 1.25; 2.50; 5.00; 10.00 µmol/l enrichments. Each point represents the mean analytical value and each error bar represents the mean value ± one standard deviation.

who use filter paper-collection devices from these two commercial sources can be assured that whole blood dried into filter paper, when properly collected, will provide a consistent volumetric measurement for analyses regardless of the FDA-registered paper source.

Future perspective

Dried blood spots have been used for infectious disease serology [8,9], drug monitoring [10,11], environmental investigations [12,13] and molecular studies [14,15]. Clinical applications using DBS and the filter paper matrix will continue to expand. DBS are often the sample of choice in resource-poor areas because of their ease of collection and storage, minimal risk of exposure to blood-borne pathogens and ability to be transported without refrigeration. As the uses of DBS evolve, so will technologies that make use of new multiplexed methods for DBS applications.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interest disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

- Filter paper collection devices offer a high degree of uniformity for whole blood specimens.
- The newborn Screening Quality Assurance Program monitors each lot of filter paper from US FDA-approved manufacturers relative to previous lots and approved standards.
- The newborn Screening Quality Assurance Program prepared dried blood spots on filter paper from two manufacturers using analyte-enriched whole blood.
- Blind-coded dried blood spots were sent to reference laboratories for testing using routine newborn screening analytical methods.
- Data demonstrated comparability of the two commercial sources of filter paper across analytes and testing methods.

Bibliography

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1 Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* 32, 338–343 (1963).

2 Guthrie R. The origin of newborn screening. *Screening* 1, 5–15 (1992).

▪ **Provides a historical perspective of the foundation for newborn screening by the man who devised the first newborn screening test on dried blood spots.**

3 De Jesús VR, Mei JV, Bell CJ, Hannon WH. Improving and assuring newborn screening laboratory quality worldwide: thirty year experience at the Centers for Disease Control and Prevention. *Semin. Perinatol.* 34, 125–133 (2010).

▪▪ **In-depth description of the quality assurance system for laboratories conducting newborn screening and the systems expansion as states added tests and adopted new technologies.**

4 Hannon WH, Whitley RJ, Davin B *et al.* Blood collection on filter paper for newborn screening programs; approved standard-fifth edition. Clinical Laboratory Standards Institute, LA5–A4, Wayne, PA, USA (2007).

▪▪ **The standard of practice for dried blood spot specimen collection and filter paper specifications.**

5 Adam BW, Alexander JR, Smith SJ *et al.* Recoveries of phenylalanine from two sets of dried blood spot reference materials: prediction from hematocrit, spot volume and paper matrix. *Clin. Chem*. 46, 126–128 (2000).

6 Mei JV, Alexander R, Adam BA, Hannon WH. Use of filter paper for the collection and analysis of human whole blood specimens. *J. Nutr.* 131, 1631S–1636S (2001).

7 De Jesús VR, Chace DH, Lim TH, Mei JV, Hannon WH. Comparison of amino acids and acylcarnitines assay methods used in newborn screening assays by tandem mass spectrometry. *Clin. Chim. Acta.* 411, 684–689 (2010).

8 Gwinn M, Pappaioanou M, George JR *et al.* Prevalence of HIV infection in childbearing women in the United States. Surveillance using newborn blood samples. *JAMA* 265, 1704–1708 (1991).

9 Mercader S, Featherstone D, Bellini WJ. Comparison of available methods to elute serum from dried blood spot samples for measles serology. *J. Virol. Meth.* 137, 140–149 (2006).

10 Garcià-Lerma1 JG, McNulty A, Jennings C, Huang D, Heneine W, Bremer JW. Rapid decline in the efficiency of HIV drug resistance genotyping from dried blood spots and dried plasma spots (DPS) stored at 37°C and high humidity. *J. Antimicrob. Chemother.* 64, 33–36 (2009).

11 Koal T, Burhenne H, Römling R *et al.* Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 19, 2995–3001 (2005).

12 Olshan AF. Meeting report: the use of newborn blood spots in environmental research: opportunities and challenges. *Environ. Health Perspec.* 115, 1767–1769 (2007).

13 Otero-Santos SM, Delinsky AD, Valentin-Blasini L *et al.* Analysis of perchlorate in dried blood spots using ion chromatography and tandem mass spectrometry. *Anal. Chem.* 81, 1931–1936 (2009).

14 Walter J, Kuhn L, Semrau K *et al.* Detection of low levels of human immunodeficiency virus (HIV) may be critical for early diagnosis of pediatric HIV infection by use of dried blood spots. *J. Clin. Microbiol.* 47, 2989–2991 (2009).

15 Cordovado SK, Earley MC, Hendrix M *et al.* Assessment of DNA contamination from dried blood spots and determination of DNA yield and function using archival newborn dried blood spots. *Clin. Chim. Acta.* 402, 107–113 (2009).

■ Website

101 Centers for Disease Control and Prevention. Laboratory quality assurance and standardization programs www.cdc.gov/laboratoriestandards/nsqap_reports.html