Cost-Effective and Scalable DNA Extraction Method from Dried Blood Spots

Carlos A. Saavedra-Matiz,1* Jason T. Isabelle,1 Chad K. Biski,1 Salvatore J. Duva,1 Melissa L. Sweeney,2 April L. Parker,1 Allison J. Young,1 Lisa L. DiAntonio,1 Lea M. Krein,1 Matthew J. Nichols,1 and Michele Caggana1

BACKGROUND: Dried blood spot (DBS) samples have been widely used in newborn screening (NBS) for the early identification of disease to facilitate the presymptomatic treatment of congenital diseases in newborns. As molecular genetics knowledge and technology progresses, there is an increased demand on NBS programs for molecular testing and a need to establish reliable, low-cost methods to perform those analyses. Here we report a flexible, cost-efficient, high-throughput DNA extraction method from DBS adaptable to small- and large-scale screening settings.

METHODS: Genomic DNA (g.DNA) was extracted from single 3-mm diameter DBS by the sequential use of red cell lysis, detergent-alkaline, and acid-neutralizing buffers routinely used in whole blood and plant tissue DNA extractions. We performed PCR amplification of several genomic regions using standard PCR conditions and detection methods (agarose gel, melting-curve analysis, TaqMan-based assays). Amplicons were confirmed by BigDye® Terminator cycle sequencing and compared with reference sequences.

RESULTS: High-quality g.DNA was extracted from hundreds of DBS, as proven by mutation detection of several human genes on multiple platforms. Manual and automated extraction protocols were validated. Quantification of g.DNA by Oligreen® fluorescent nucleic acid stain demonstrated a normal population distribution closely corresponding with white blood cell counts detected in newborn populations.

CONCLUSIONS: High-quality, amplifiable g.DNA is extractable from DBSs. Our method is adaptable, reliable, and scalable to low- and high-throughput NBS at low cost ($0.10/sample). This method is routinely used for molecular testing in the New York State NBS program.

The universal collection, transport, and use of dried blood spots (DBSs)3 made population screening of newborns possible and facilitated the creation of newborn screening programs (NBSPs) (1). A DBS is prepared by spotting a small aliquot of blood obtained by heel or finger stick onto 903® filter paper. The 903 paper, an FDA-registered in vitro class II medical device, is the international standard for NBS and is manufactured from 100% pure cotton linters (2). Cotton fibers are >90% cellulose. The simplicity and low cost of sample collection, transport, and storage and the stability of specific biomarkers (3) made the DBS the specimen of choice for NBS and an important sampling mechanism for clinical and basic research in fields ranging from pediatrics to medical anthropology and environmental studies (3–5). Guthrie cards or DBSs represent an important population-based resource containing biochemical, immunological, environmental, and genomic information that is important from the public health perspective. Molecular tests are increasingly being added by NBSPs both as a confirmatory (second-tier) and primary (first-tier) screening tests (6–8), creating a need for a reliable, inexpensive, and practical DNA extraction method from DBSs that performs with high efficiency regardless of sample load. Several natural components of blood, such as hemin and immunoglobulins, and added anticoagulants such as EDTA and heparin, are known inhibitors of the PCR (9). Most commercially available kits were developed to extract DNA from whole blood, and the few available methods to extract DNA from a DBS are ex-
pensive and labor intensive, making their use impractical for processing a large number of samples in population-based studies for NBS. We report here a simple, efficient, low-cost, adaptable, and reliable method to extract DNA from DBSs in either a manual or automated format. This method, named CASM (first author’s initials), relies on the treatment of the DBS as a hybrid animal–plant tissue (whole blood–cotton/cellulose), allowing for the use of a combination of whole blood and plant DNA extraction methods (10, 11).

Materials and Methods

DNA EXTRACTION

This study was approved by the institutional review board at the New York State Department of Health (NYSDOH). Anonymous 3-mm dried blood spot punches were removed from residual NBS samples and placed into the wells of a 96-well PCR plate by using clean forceps or a Delfia® DBS puncher. Red blood cell lysis buffer (RBC-LB) (0.01 mol/L Tris-HCl, 320 mmol/L sucrose, 5 mmol/L MgCl2, 1% Triton × 100) adjusted to pH 8.0 and filtered with a Nalgene® 0.2-µm pore size filter (150 µL) was added to each well. The plate was centrifuged briefly at 1140 g (2500 rpm) in a Beckman Allegra 6R centrifuge to ensure that all DBSs were immersed in the RBC-LB. The plate was vortex mixed at speed 1 for 10 min on a Vortex-mix-Genie-2® to collect all buffer at the bottom of the well. Buffer B (100 mmol/L Tris-HCl, 2 mmol/L EDTA) (50 µL) was added to each well and the plate was covered with strip caps and quickly centrifuged at 410 g (1500 rpm) to ensure that the spots were immersed in the buffer. Each plate was incubated at 95 °C for 12 min in a thermocycler or a heat block and held at 4 °C after incubation. The plate was washed with shaking for 5 min in 80 µL dH2O boiled DBS eluate were discarded after each wash. Buffer A (100 mmol/L NaOH, 2% Tween® 20) (50 µL), prepared fresh from 10 mol/L NaOH and 20% Tween 20 stock solutions, was added to each well and the plate was covered with strip caps and quickly centrifuged at 410 g (1500 rpm) to ensure that the spots were immersed in the buffer. Each plate was incubated at 95 °C for 12 min in a thermocycler or a heat block and held at 4 °C after incubation. The plate was washed with shaking for 5 min in 80 µL dH2O before the addition of RBC-LB (100 µL). Each subsequent wash used 80 µL of dH2O. Incubation in buffer A was performed in a shaker/heater (700 rpm/70 °C) Peltier microplate for 12 min, followed by an additional 2 min at 27 °C.

PRIMERS AND PCR CONDITIONS

Primers flanking exonic and promoter regions of the genes galactosylceramidase (GALC),4 medium and very long chain acyl-CoA dehydrogenases [acyl-CoA dehydrogenase, C-4 to C-12 straight chain (ACADM) and acyl-CoA dehydrogenase, very long chain (ACADVL)], galactose-1-phosphate uridyl transferase (GALT), and phenylalanine hydroxylase (PAH) were designed in the NYS NBS program using the Primer3™ free software available at http://frodo.wi.mit.edu/primer3/.

Genomic DNA (g.DNA) (1–5 µL) extracted from DBS was used for the optimization of standard and multiplex PCR amplifications followed by BigDye sequencing of the GALC, ACADM, ACADVL, GALT, and PAH genes. We also tested a TaqMan assay for the quantification of T-cell receptor excision circles (TRECs) for the screening of severe combined immunodeficiency (SCID) using standard conditions.

All amplifications were carried out on thermal cyclers (MJ Research PTC-200, Bio-Rad DNAEngine®, Applied Biosystems GeneAmp® PCR System 9700). Quantitative real-time PCR (q-RT-PCR) and TaqMan assays were performed on a Roche LightCycler®, a LightCycler 480, and an Applied Biosystems 7900HT fast RT-PCR System.

Results

PCR INHIBITION

To demonstrate the PCR-inhibitory effect of increasing DBS eluate concentrations, a 1:100 dilution of plasmid (clone AGS 8–18) containing the fusion-gene g.30kb deletion (844 bp) of the CYP21 gene responsible for congenital adrenal hyperplasia (12) was amplified. Increasing volumes of a dH2O boiled DBS eluate were added to each reaction (Fig. 1). There was a progressive reduction of PCR product from the plasmid as the input DBS eluate increased (lanes 2–6). Lane 7 confirmed the efficiency of the PCR reaction by showing amplification of a minimal amount of plasmid (tip touch from the previously inhibited reaction) without eluate inhibition.

4 Human genes: GALC, galactosylceramidase; ACADM, acyl-CoA dehydrogenase, C-4 to C-12 straight chain; ACADVL, acyl-CoA dehydrogenase, very long chain; GALT, galactose-1-phosphate uridyl transferase; PAH, phenylalanine hydroxylase; FCGR2A, Fc fragment of IgG, low affinity IIa, receptor (CD32); RPP30, ribonuclease P/MRP 30kDa subunit.
PCR AMPLIFICATION
To demonstrate the quality of the CASM-extracted g.DNA, we targeted different genomic regions for amplification (Fig. 2). Fig. 2A shows the amplification of the mutant (462-bp) and normal (754-bp) alleles of the most common (g.30Kb del) deletion of the GALC gene causing Krabbe disease (7). Single-allele reactions were carried out in a total volume of 40 μL using 2 μL of extracted g.DNA as template. Fig. 2B shows the successful amplification of a 371-bp PCR product of the Fc fragment of IgG, low affinity IIa, receptor (CD32) (FCGR2A) gene in 18 different anonymous DBSs. Fig. 2C demonstrates the integrity of the g.DNA. Amplification of larger genomic fragments necessary to sequence the ACADVL gene (1.7, 0.8, and 1.0 kb) was routinely done. Fig. 2C shows the results for 2 DBSs. Reactions for the FCGR2A and ACADVL genes were carried out in a total volume of 50 μL using 5 μL of extracted DNA as template.

QUANTITATIVE REAL-TIME PCR
The quality of extracted g.DNA from DBS is also shown in Fig. 3. In routine clinical screening in NYS, g.DNA is reproducibly extracted from DBS. For example, only 0.5% amplification failures occur when DNA extracted by this method is used as template for the quantitative real time TaqMan (first tier; approximately 1000 samples daily) assay used to detect TREGs for 374 samples used in the laboratory for SCID screening. These results were obtained after automated DNA extraction on a Biomek NX. Fig. 3B shows the results of qPCR-FRET detection used for the identification of 1 individual who was heterozygous and 2 who were homozygous for a common variant of the GALC gene. Fig. 3C shows BigDye sequence analysis for the c.286A>G (p.Thr96Ala) mutation in exon 4 of the GALC gene.

DNA QUANTIFICATION AND EXTRACTION EFFICIENCY
Screening programs have little to no control over the quality of the samples received. Further, the number white blood cells (WBCs) in healthy newborns range from 4000 to 30 000/μL of whole blood (13), with a mean of approximately 17 000 WBCs/μL. The effi...
ciency of any DNA extraction method in a screening setting must be high enough to prevent assay failure for infants on the low end of the reference interval for WBCs. A 3-mm DBS contains 3.1 μL of whole blood (14) and 1 cell yields approximately 6.6 pg of g.DNA. For a mean of 17 000 WBC/μL, an extraction with 100% of efficiency is expected to yield 3.47 ng/μL of g.DNA per 3-mm DBS.

Quantifying DNA from every DBS for routine screening is impractical. DNA yields from DBSs are too

Fig. 3. qRT-PCR and Sanger sequencing.

(A), Multiplexed TagMan assay in a 384-well format with 2-probe detection with a 7900HT fast RT-PCR platform from Applied Biosystems. Amplifications were carried out in a total volume of 10 μL with 2 μL g.DNA input per reaction. (B), qPCR-FRET detection of the common c.1637T>C (p.Ile546Thr) polymorphism of the GALC gene. Amplifications were carried out in a total volume of 20 μL using 2 μL of g.DNA as template. (C), BigDye sequence analysis showing an A-to-G transition in exon 4 of the GALC gene (amplicon size = 400 bp). PCR products were purified with ExoSap and cycle sequenced according to standard manufacturer recommendations. ΔRn, baseline-corrected normalized reporter signal.

Continued on page 1049
low to allow reliable quantification by 260/280 absorbance measurements (15). Quantification of such low concentrations in these types of “crude” extractions has a large margin of error, most often artificially inflating the DNA concentration. To estimate the extraction efficiency, the Oligreen fluorescent assay (Molecular Probes) was used to quantify DNA from 160 randomly selected DBSs, and we converted this concentration to WBC counts using the assumptions cited above. Fig. 4 shows a normal distribution of WBC counts as expected from a random newborn population and a theoretical yield of 6.6 pg of DNA per cell with a mean of 15 500 WBC/μL predicted, assuming an extraction efficiency of >90% (3.17 ng/μL and 91.4% recovery). Similar values were obtained from the manual and automatic extraction methods. A similar pattern was observed when RPP30 and TREC were quantified by the TaqMan assay (data not shown).

To increase the DNA yield, multiple spots were individually processed up to the second dH2O wash and combined before the buffer A boiling step. For the remaining steps these spots were treated as if they were 1 spot. DNA quantification was performed with the Oligreen fluorescent nucleic acid stain assay (Molecular Probes). Fig. 5 shows a consistent increase of DNA concentrations as more DBSs are added per individual. This is effective for samples with low DNA yield from a single sample (e.g., individual 2 in Fig. 5).

This multispot version was tested in anticipation of using platforms such as microarrays and next-

---

**Fig. 4.** Distribution of g.DNA concentrations (ng/μL) found in 160 random DBSs and extrapolation to equivalent counts of WBCs (WBC × 1000/μL).

Numbers on top of bars are the mean g.DNA concentration per range converted to WBC counts. Bars correspond to the number of individuals found with g.DNA concentrations within each range.
generation sequencing. Preliminary results from Illumina HumanOmni1-Quad_v1–0_B bead arrays showed >99% single-nucleotide polymorphism (SNP) call rates. Furthermore, preliminary data obtained with the Ion Torrent platform also demonstrated good sequence data (Table 1), high SNP rate calls, and >99% concordance with Sanger sequencing.

Discussion

As molecular testing becomes routine in screening programs around the world, a simple, low-cost, reliable, and scalable method for extracting amplifiable g.DNA from DBSs is necessary. Several methods ranging from microextraction to autoclaving and overnight methanol fixation have been described \((16 – 18)\). However, these laborious and/or lengthy processes are not practical in a screening setting where hundreds of samples per day are processed.

The CASM method takes advantage of the hybrid nature of the DBS. The method is simple, adaptable, inexpensive, scalable, and efficient and can meet the needs of a small research laboratory or a large NBSP. Improvements include a wash step to remove PCR inhibitors in whole blood by using traditional animal/human RBC-LB \((10)\). DNA is extracted from the WBCs trapped in the cotton/cellulose mesh and released from the fiber paper using a detergent–alkaline solution that reportedly macerates plant tissue \((11)\) without diminishing DNA quality. Although DBS samples stored <2 years can be used in the protocol as described, older (drier) spots are easily extracted by using a 10-min preincubation in a \(\text{dH}_2\text{O}\) wash step to facilitate the full absorption of the initial RBC-LB. The flexibility of the method makes it easy to adapt to specific laboratory/program needs. Our laboratory has successfully used 1–2 \(\mu\)L from a 1:4–1:16 dilution of the original eluate \((100 \mu\)L) for different PCR protocols, providing enough g.DNA for hundreds of reactions. The g.DNA concentration obtained from most DBSs ranges from 1.0–6.0 ng/\(\mu\)L or 100–600 ng total, as is expected from the normal WBC distribution of a random population (Fig. 4), allowing for multiple dilutions and single or multiple gene targets. This is advantageous for NBS, for which the number of tests performed is limited by the amount of sample. This method is not only versatile and scalable but also is cost-effective and ideal for molecular NBS and small/large laboratories performing molecular population-based research and medical anthropology in the field. The estimated cost of this method is $0.10/sample. The NYS NBSP has used the method for more than 5 years to extract g.DNA for BigDye terminator (Applied BioSystems) cycle sequencing of the complete \(GALC\) gene (Fig. 3C) and qRT-PCR for the most common known mutations of the \(GALT\) and \(ACADM\) genes; these assays are presently used as second-tier (reflex) tests in the NYS NBSP. This g.DNA is successfully used in different qualitative and quantitative (SNP analysis, \(RPP30\), TRECs) qRT-PCR assays and platforms, which strongly suggests the presence of intact functional DNA \((15)\). We have also used this method to validate complete gene sequencing for the \(ACADVL\), \(GALT\), and \(PAH\) genes and plan to incorporate these genes as reflex tests in our program.

We have developed an automated version to screen for SCID \((8)\) in newborns as a first-tier test by detecting TREC copies on more than 1000 \(\times\) 3-mm DBS per day. Lastly, high SNP calling rates (>99%) have been obtained on the Illumina HumanOmni1-Quad_v1–0_B bead arrays. Similarly, preliminary experiments with bar-code amplicon sequences of 5
genes and 5 different individuals have indicated that high library and depth coverage (Table 1) and Sanger concordance (>99%) are achievable on the Ion Torrent NextGen sequencing platform.

In summary, the CASM method results in a very efficient, reliable, flexible, cost-effective, and scalable DNA extraction method from DBS, suitable for low- and high-throughput methods in research and screening laboratories.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

References


Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: C.A. Saavedra-Matiz, New York State Department of Health, Newborn Screening Program; J.T. Isabelle, New York State Department of Health; C.K. Biski, New York State Department of Health; S.J. Duva, New York State Department of Health; A.J. Young, New York State Department of Health; L.L. Di-Antonio, New York State Department of Health; L.M. Krein, New York State Department of Health.

Consultant or Advisory Role: None declared.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: New York State Department of Health.

Expert Testimony: None declared.

Patents: None declared.

Other: M. Caggana, Association for Public Health Laboratories.

Role of Sponsor: No sponsor was declared.

Clinical Chemistry 59:7 (2013) 1051