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Genotypic Confirmation from the Original Dried Blood Specimens in a Neonatal Hemoglobinopathy Screening Program

MARIA DESCARTES, YING HUANG, YAO HUA ZHANG, LINDA L. McCABE, RICHARD GIBBS, BRADFORD L. THERRELL, JR., AND EDWARD R. B. McCABE

Institute for Molecular Genetics [M.D., Y.H., Y.H.Z., L.L.M., R.G., E.R.B.M.] and Department of Pediatrics [E.R.B.M.], Baylor College of Medicine, Houston, Texas 77030, and the Texas Department of Health, Austin, Texas 78756 [B.L.T.]

ABSTRACT. Dried blood spots are used for newborn screening because of ease of sample collection, handling, and shipment. DNA is stable and accessible in the filter paper matrix. Genotypic confirmation using initial specimens is demonstrated for a regional screening program. Seventy-five blinded samples underwent DNA analysis after Hb electrophoresis. DNA was microextracted from a ½-inch semicircle (25 μL whole blood equivalent), amplified, and analyzed by four different methods. Direct amplification without microextraction and automated sequencing from microextracted DNA also was performed. All four analyses agreed for the A and S alleles in 70 of 75 specimens. Three disagreements were clarified by the other semicircle from the original sample: two were due to polymerase chain reaction contamination and one to contamination of one of four analytical tests. Two would have required analysis of a second specimen, one because of polymerase chain reaction failure and the second because the patient had S/β-thalassemia. Direct amplification without microextraction was successful in an additional 77 of 78 specimens for analysis of the A, S, C, and E alleles. Automated direct sequencing from microextracted DNA was demonstrated for the A, S, and C alleles. Analysis of microextracted DNA from dried blood specimens for A and S alleles reduced the need for and costs of obtaining a second specimen for confirmation by 97%. Direct amplification without microextraction for analysis of A, S, and C alleles permits additional reduction in personnel time and costs. We have demonstrated that microextracted DNA is amenable to automated sequencing after asymmetric polymerase chain reaction. Direct genotypic confirmation can facilitate diagnosis and initiation of medical intervention. (*Pediatr Res* 31: 217–221, 1992)

Abbreviations

PCR, polymerase chain reaction
PKU, phenylketonuria
CF, cystic fibrosis
ASO, allele specific oligonucleotide
HRP, horseradish peroxidase

The collection of newborn screening specimens on filter paper blotters represented a major innovation that facilitated the establishment and regionalization of screening programs (1, 2). These screening programs were originally developed for PKU, but are incorporating an expanding group of disorders, including the hemoglobinopathies (3). With rare exceptions, these expanded test batteries have continued to use dried blood specimens in a filter paper matrix because of the ease of sample collection, handling, and shipment and the stability of the analytes. Typically, the analytes evaluated by neonatal screening laboratories have included metabolites such as phenylalanine for PKU, or galactose and galactose-1-phosphate for galactosemia; hormones such as thyroid hormone and thyroid stimulating hormone (TSH) for congenital hypothyroidism, or 17-hydroxyprogesterone for congenital adrenal hyperplasia; and proteins such as galactose-1-phosphate uridyl transferase for galactosemia, immunoreactive trypsinogen for CF, or Hb for the hemoglobinopathies (1, 3, 4).

More recently, it has become evident that DNA is also a stable and accessible analyte in dried blood specimens (5–21). Because the individual's DNA is present in all nucleated cells, any mutations represented in an individual can be detected using the dried sample of peripheral blood without regard for tissue or developmental stage specific expression of the phenotype. This latter characteristic is valuable for a hemoglobinopathy screening program, because the adult Hb genes are submaximally expressed at birth and in the perinatal period. Direct genotyping from the original newborn blotter would permit early and rapid confirmation of positive screening tests, clarification of equivocal results, and effective initiation of penicillin prophylaxis to prevent mortality related to overwhelming infection in patients diagnosed with sickle cell disease (10, 22, 23). Previous studies have demonstrated the possibility of DNA follow-up for hemoglobinopathy screening (10, 11). In this report, we describe the results of a study evaluating the integration of direct genotypic confirmation with a regional, protein-based, neonatal screening laboratory. This study examines the validation of DNA analyses in this setting and the influence of the DNA confirmatory laboratory on requirements for repeat specimens. In addition, we provide results demonstrating the feasibility of improving the automation and cost-effectiveness of DNA follow-up.

MATERIALS AND METHODS

Specimens. During the period from September 18 to October 12, 1989, 75 samples were sent from the state newborn screening laboratory at the Texas Department of Health to the DNA follow-up laboratory at Baylor College of Medicine. These samples were single ½-inch circles cut from neonatal specimen blotters (no. 903 specimen collection paper, Schleicher & Schuell,

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Correspondence and reprint requests: Edward R. B. McCabe, M.D., Ph.D., Institute for Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, S-921, Houston, TX 77030.
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Inc., Keene, NH) after all routine screening tests had been performed. The identity and electrophoretic results of these were unknown to all of the personnel in the DNA laboratory. The results from the state screening laboratory were revealed only after the DNA genotype had been determined.

DNA microextraction. A ½-inch semicircle was cut from each specimen circle of dried blood using stainless steel scissors that were routinely washed in 0.3 N HCl for 10 min between samples to depurinate DNA and prevent cross-contamination of specimens (10). Each semicircle represented the dried equivalent of approximately 25 μL of whole blood (5, 10, 24). The microextraction procedure was similar to that reported previously (10), with the exception that the proteinase K digestion was extended from 2 to approximately 16 h (overnight) to improve DNA yield. The procedure included methanol fixation, SDS and proteinase K treatment, phenol extraction (three times), chloroform-isopropyl alcohol extraction (two times), ethanol precipitation, and drying the precipitated DNA. After dissolving the DNA in 100 μL TE (10 mM Tris HCl and 1 mM EDTA, pH 8.0), the quality was evaluated and the concentration was estimated by electrophoresis of a 10-μL aliquot with standards of known concentration on a 1% agarose (Sea Kem ME) gel that was stained with ethidium bromide. The DNA median yield was 1500 ng/semicircle (range 900–2000 ng/semicircle) or approximately 60 ng/μL dried equivalent of whole blood (range 36–80 ng/μL).

Amplification. An aliquot of DNA (generally 200–300 ng or 10–20% of the microextracted material) was taken for amplification using 2.5 U *Thermus aquaticus* (*Taq*) polymerase (Cetus Corp., Emeryville, CA), and reaction buffer and other components as previously described (10), for a reaction volume of 100 μL. A DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) was used for 30 cycles of amplification: denaturation 94°C for 0.5 min, annealing 55°C for 0.5 min, and extension 72°C for 1.0 min. Two primers flanking a 299-bp genomic region containing the sickle cell mutation were used: PCO3 (25) and PMC1110 (5′-CTCAAAGAACCTCTGGGTCC-3′).

Amplification without microextraction. Although the method described above for microextraction and amplification was used for all of the original 75 blinded specimens obtained from the Texas Newborn Screening Laboratory in Austin, subsequently we adapted a method from Schwartz *et al.* (26) and previous work on boiling of specimens from our group (10) that avoided the need for manual microextraction. Aliquots measuring approximately 4 × 4 mm (representing the dried equivalent of approximately 8 μL whole blood) were cut from each dried blood specimen and placed in 1.5-mL microfuge tubes to which the complete amplification cocktail including primers, and in some cases *Taq* polymerase, were added as described above. These microfuge tubes were then placed in the automated thermal cycler programmed with an initial 15-min step at 95°C followed by 30 cycles of amplification described above, modified to increase the 94°C denaturation step from 0.5 to 1.0 min. If *Taq* polymerase had not been added before the initial 15-min cycle, it was added after this step and before the 30-cycle amplification program was initiated. After amplification, the tubes were centrifuged and the supernatants were removed and aliquoted for analysis.

Analysis. Four detection methods were used to analyze the amplified products in the blinded assessment of samples from the state screening laboratory. Each method used 10–20 μL of the 100-μL PCR reaction volume. Two of these methods were based on dot or slot blot hybridizations of amplified DNA with ASO probes (p19A and p19S) (27), either labeled with radioactive phosphorus (^{32}P) (10) or HRP (12, 25). The third approach involved direct digestion of the PCR amplified products with *Dde*I and separation of the restriction fragments by agarose electrophoresis in a method adapted from Kazazian (28). The fourth method used Southern blotting (29) and hybridization with ^{32}P-labeled ASO. Routine controls included patients' samples of known genotypes AA, AS, and SS for the blinded study. A reagent blank controlled for contamination of the assay components.

Products of the direct amplification reactions were analyzed for the A, S, C, and E alleles using dot-blot or slot-blot hybridizations with radiolabeled ASO probes. The A, S, and C ASO were 19-mers previously described (p19A, p19S, and p19C) (27), and the E ASO was a 20-mer, which we have designated p20E (5′-AAGTTGGTGGTAAGGCCCTG-3′).

Automated direct sequencing. Microextracted DNA was amplified using oligonucleotide primers flanking the mutation site (no. 203, 5′-GACACAACTGTGTTCACTAGCAACC-3′; no. 1180, 5′-ATCCTGAGACTTCCACACTGATGCA-3′). Fluorescent DNA sequence primers were prepared and fluorescent DNA sequencing carried out according to Gibbs *et al.* (30) using the ABI Automated Sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Each of the 75 specimens was amplified and analyzed by all four DNA methods. Figure 1 shows an example of the results obtained after hybridization of a slot blot with radiolabeled ASO for the A and S alleles. Each group of unknowns was accompanied by known AA, AS, and SS DNA specimens (Fig. 1, *lower portion*). A typical dot-blot hybridization with HRP-labeled ASO is shown in Figure 2, with the controls in the lower right portion of each dot blot. After direct *Dde*I digestion of the PCR-amplified products, these were electrophoretically separated and stained with ethidium bromide as shown in Figure 3 (controls on the *right*). Southern blots of undigested PCR products were hybridized with radiolabeled ASO probes identical to those used for the dot- or slot-blot hybridizations (results not shown).

When the results of the state laboratory Hb electrophoresis and the initial results from the DNA laboratory were compared,

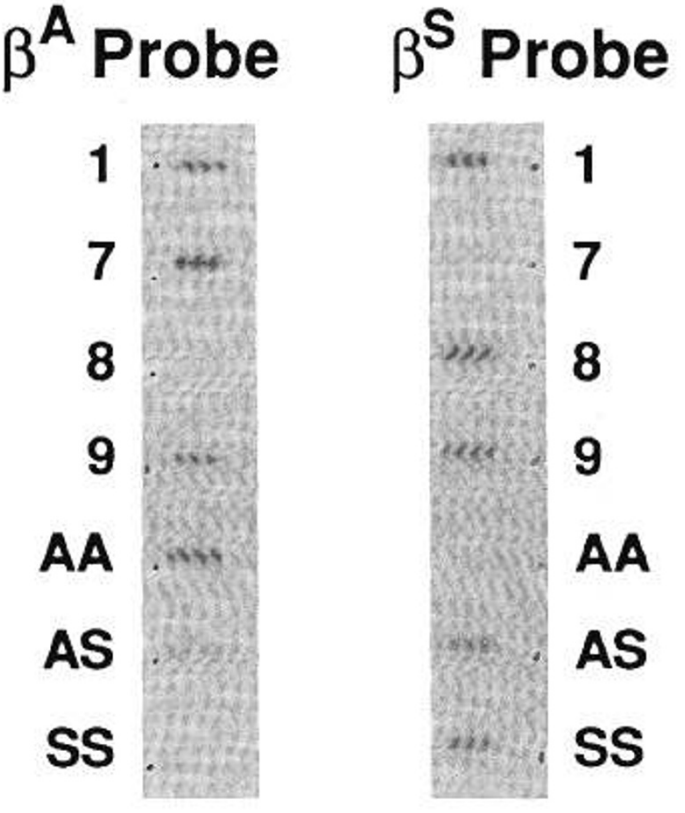

Fig. 1. Slot-blot hybridization with radiolabeled β^A and β^S ASO probes. The slot blot on the *left* was hybridized with the β^A probe and that on the *right* with the β^S probe. The numbered samples represent DNA from unknowns, and the control samples are at the *bottom* of each slot-blot autoradiogram.

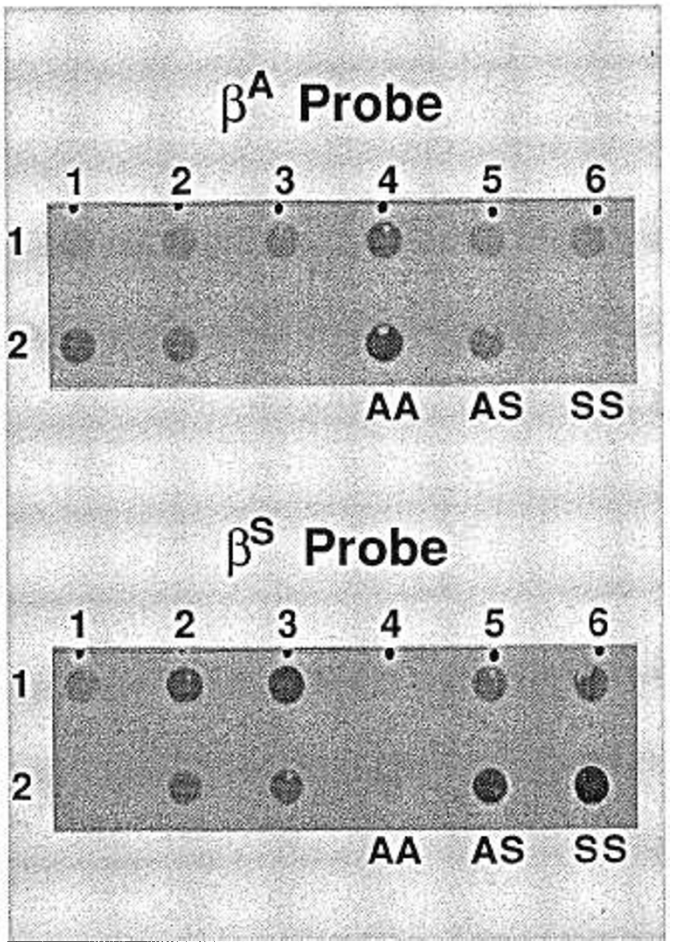

Fig. 2. Dot-blot hybridization with HRP-labeled β^A and β^S ASO probes. The dot blot on the *top* was hybridized with the β^A probe and that on the *bottom* with the β^S probe. The control samples are at the *lower right* of each dot blot and the other specimens are unknowns. The HRP-coupled probes give blue signals on the original dot blots.

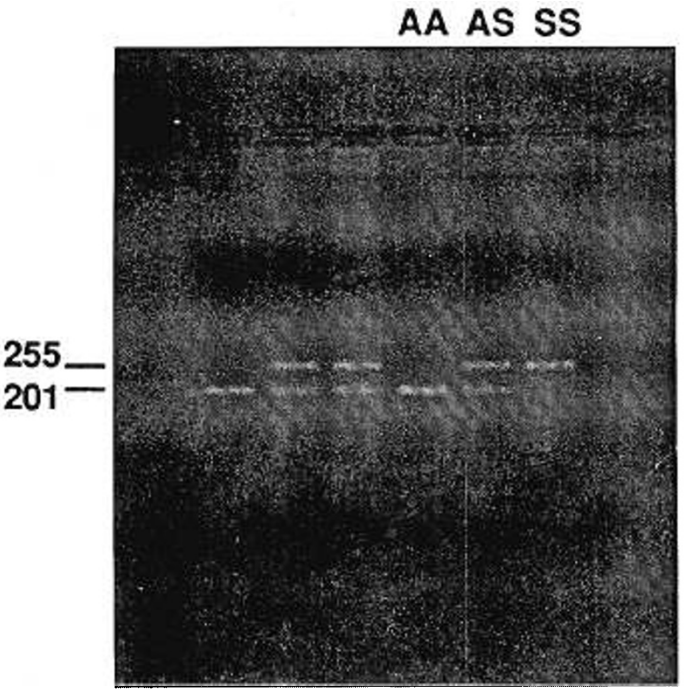

Fig. 3. Electrophoretic separation after digestion of amplified DNA specimens. The DNA samples are amplified by PCR and digested with *Dde*I. The digestion products were separated by electrophoresis on 2% agarose (Sea Kem ME), and the gel stained with ethidium bromide and photographed on a UV-light box. Control samples are shown in the *right three lanes*. The 255-bp product is the uncut band characteristic of the β^S allele and the 201-bp product is one digestion product seen with the β^A allele. The other product of the β^A allele digestion is a 54-bp fragment, and there is also a constant 44-bp piece, neither of which is well visualized here.

Table 1. *Comparison of results from state and DNA laboratories*

State	No.	Agreement	Disagreement	Sample no.
FA	30	27/30	3/30	42, 45, and 47
FAS	30	30/30	0/30	
FS	15	13/15	2/15	2 and 61
	75	70/75	5/75	

there was complete agreement in 70 of the 75 specimens (Table 1). The five samples that showed a disagreement were numbers 2, 42, 45, 47, and 61. When samples 42, 45, and 61 were repeated in the DNA laboratory using the original specimens, the results agreed with the state laboratory. For samples 42 and 45, the state laboratory results were FA and the original DNA laboratory results were AS by all four methods. When 42 and 45 were reextracted in the DNA laboratory, the results were AA on all four methods. This suggests that the initial results for 42 and 45 were due to contamination during the first DNA microextraction or the initial PCR amplification. It is interesting to note that 42 and 45 were in the same batch during their initial testing in the DNA laboratory. For sample 61, three of the original tests in the DNA laboratory showed SS in agreement with the state laboratory result of FS. The fourth DNA analysis on sample 61, using the dot blot HRP-coupled ASO, showed AS. Repeating the analysis on sample 61 using the remaining specimen showed FS for all four methods in the DNA laboratory. This suggested that the initial disagreement was due to contamination at the dot-blot step after aliquoting the amplification products.

To resolve the disagreement for samples 2 and 47, we requested a repeat specimen from the state laboratory. The initial testing of sample 47 gave inadequate amplification of the microextracted DNA. Analysis was attempted by HRP-coupled ASO and Southern blotting, and although the results were suboptimal they were interpreted as AS. The state laboratory obtained FA. On the repeat specimen, the microextracted DNA again did not support amplification; no analyses were successful from the repeat circle. Both the original and repeat circles of blood from sample 47 were unacceptable, and in routine practice a repeat sample would have been requested from the patient before any genotypic results could be reported. For sample 2, the state laboratory obtained an FS result, while the DNA laboratory showed AS on all four tests on both the original and the repeat specimen. We suggested to the state laboratory that these results would be consistent with a disorder such as S/β-thalassemia. Additional laboratory testing confirmed this diagnosis for the patient. This discrepancy between the state laboratory and the DNA laboratory was real, and resulted in clinically useful information.

After analysis of these 75 specimens, we modified the procedure to use a 16-mm^2 specimen, representing the dried equivalent of approximately 8 μL whole blood, for direct amplification without microextraction. The automated amplification program was altered by introducing an initial 15-min step at 95°C and increasing the length of the 94°C denaturation step to 1 min. We have had successful direct amplification in 77 of 78 specimens, which were analyzed for the A, S, C, and E alleles using dot-blot hybridization with radiolabeled probe.

Automated direct sequencing after asymmetric PCR was performed successfully on DNA microextracted from newborn screening specimens representing samples with the A, S, and C alleles (Fig. 4). Heterozygotes were detected by the presence of

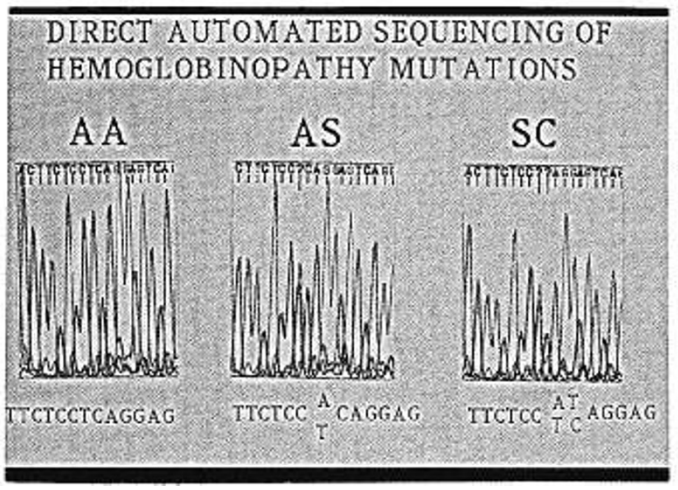

Fig. 4. Direct automated sequencing of the β-globin gene by asymmetric PCR from dried blood specimens. DNA microextracted from newborn screening specimens was amplified by asymmetric PCR and sequenced using an ABI 3710A DNA sequencer and fluorescent primers. The sequence data is shown for the antisense strands of AA DNA (*left panel*), AS DNA (*center panel*), and SC DNA (*right panel*). At positions that are heterozygous and show two different nucleotides, the sequencer indicates a question mark; the two nucleotides at that site can be identified from the peaks in that position on the graphic printout.

two different nucleotides at the same position(s) (Fig. 4, *panels AS* and *SC*).

DISCUSSION

Seventy-five newborn screening specimens were evaluated by molecular genetic analysis of the A and S alleles during a 1-mo period. There was complete agreement between the Hb electrophoresis results from the state screening laboratory and the DNA results for 70 of the 75 specimens, or 93% of those analyzed. Results from three (4%) of the specimens that disagreed between the laboratories were attributable to contamination; these three discrepancies were resolved using the remaining semicircle from the original specimen without need for a second sample. The two remaining samples, or 3% of the total group, would have required a repeat blood collection from the patient for clarification. One of these was due to inadequacy of amplification on two attempts from each of two separate circles from the original blotter. The second was due to an actual discrepancy consequent to a genetic compound between the S and a β-thalassemia allele giving an A result for the second position of the β^6 codon targeted by these diagnostic methods. This latter type of discrepancy would provide information of clinical value from the DNA follow-up program. To improve molecular genetic ascertainment of patients with S/β-thalassemia who initially are screened positive (FS) and subsequently are determined to have an AS genotype, we have developed a method using reverse transcriptase followed by PCR amplification for analysis of mRNA from the initial dried blood specimens (31).

In the absence of a DNA genotyping program, all positive and equivocal newborn screening test results would be repeated on a second liquid blood specimen drawn at 2–4 mo of age. The cost of contacting the families and acquiring the follow-up specimens are significant expenses that are usually not calculated into the overall cost of a newborn screening program (10). We have demonstrated that DNA genotyping using the original neonatal screening specimen is feasible and would reduce the number of second specimens required by an estimated 97%. In addition, this would provide more detailed genetic information that would permit better-informed education and counseling of these families at the initial contact, which we have suggested may improve compliance with antibiotic prophylaxis and medical management (10). Based on the results in this study, we have expanded our genotyping capabilities to include not only the A and S alleles but also the C and E alleles using radiolabeled ASO. In addition, it would be possible to expand this diagnostic ability to examine for thalassemias using an ethnicity-based algorithm (28). Because we can perform at least 30 analyses on each blood specimen using the microextraction or direct amplification procedures, the addition of the thalassemias to the neonatal DNA genotyping repertoire would be feasible.

Automation of the DNA diagnostic procedures will be essential for any large scale neonatal or other population-based screening program, such as is eventually anticipated for CF carrier screening (32). The microextraction procedure, previously described by Jinks *et al.* (10) and used in the study of the 75 unknown specimens in this report, is extremely personnel intensive and, therefore, would be a costly component of any high-volume DNA diagnostic program based on dried blood spots (33). The procedure described by Schwartz *et al.* (26) for direct amplification from a small piece cut from a Guthrie card, without microextraction, would decrease dramatically the personnel investment required for specimen processing. This method was based on the demonstration that DNA could be amplified after boiling the dried blood specimens (10) or directly by the thermostable DNA polymerase from *Thermus thermophilus* (26, 34) and was adapted for use with *Taq* polymerase. This adaptation permits the reduction of sample size from a ½-inch diameter semicircle, representing the dried equivalent of approximately 25 μL whole blood, to a 4 × 4 mm square containing approximately 8 μL dried whole blood equivalent. This new procedure of direct amplification without microextraction also eliminates the need for, and disposal of, the toxic material, phenol, resulting in additional cost savings. This adaptation offers an advantage over the earlier boiling procedure (10), because the filter paper matrix remains in the amplification tube and does not necessitate DNA extraction from the matrix. Therefore, a portion of the DNA may be amplified *in situ*, as has been reported for crude cell lysates immobilized on nylon membranes (35).

The cost of DNA genotyping after direct amplification is estimated at $5–10 per sample. This estimate is based on a reagent expense of approximately $2 or less per specimen. A technician would be able to analyze 500–1000 samples per month, resulting in a labor cost of $2–4 per specimen. Therefore, we feel that $5–10 for analysis of a specimen represents a conservative estimate of the cost and compares favorably with protein-based confirmatory testing.

Before direct sample amplification without microextraction, DNA preparation was the rate-limiting step in the procedure. However, the analysis now becomes the limiting factor for sample throughput. We have demonstrated that analysis may be automated using direct automated sequencing by asymmetric PCR. As the cost of automated DNA sequencing is reduced in the course of the human genome initiative, such an approach will become more feasible, especially for examination of regions containing a number of different alleles. A dedicated sequencing apparatus robotically interfaced with equipment for automated amplification would be one potential technologic configuration and would have the advantage of flexibility in the analytical approach (36). Chehab and Kan (37) have demonstrated that color amplification can differentiate alternative alleles at a specific nucleotide position. This methodology, which is based on competitive oligonucleotide priming (38), also has the potential for automation. Innovation in the design and implementation of automated DNA analysis will be required for any large volume molecular genetic screening activity.

Dried blood specimens afford the same advantages for DNA analysis that they have demonstrated for over 25 years in neonatal screening: ease of specimen collection and transport to a centralized laboratory and stability of the analyte (1, 2). These attributes have been used not only for genotyping in the hemoglobinopathies (10–14), but also for PKU (6, 7, 26), CF (8, 9,

18, 19), Duchenne muscular dystrophy (15–17), medium-chain acyl CoA dehydrogenase deficiency (20), and hereditary fructose intolerance (21). Experience is showing that these specimens can be used for the same molecular genetic analyses that have been applied to liquid blood specimens.

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