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# DIAGNOSIS OF SICKLE CELL ANEMIA AND β-THALASSEMIA WITH ENZYMATICALLY AMPLIFIED DNA AND NONRADIOACTIVE ALLELE-SPECIFIC OLIGONUCLEOTIDE PROBES

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**Abstract** We have developed a simple and rapid nonradioactive method for detecting genetic variation and have applied it to the diagnosis of sickle cell anemia and β-thalassemia. The procedure involves the selective amplification of a segment of the human β-globin gene with oligonucleotide primers and a thermostable DNA polymerase, followed by hybridization of the amplified DNA with allele-specific oligonucleotide probes covalently labeled with horseradish peroxidase. The hybridized probes were detected with a simple colorimetric assay.

We demonstrated the usefulness of this method in a retrospective analysis of two pregnancies at risk for β-thalassemia and one at risk for sickle cell anemia, as well as in an analysis of nine DNA samples simulating three family sets. (N Engl J Med 1988; 319:537-41.)

SICKLE cell anemia and β-thalassemia are inherited hematologic disorders that are widespread in regions of the world where malaria was once endemic.[1] Both these autosomal recessive diseases are caused by mutations in the β-globin gene, a gene encoding a major protein component of hemoglobin A. These mutations generally involve the replacement, insertion, or deletion of one to four nucleotide bases from the DNA sequence of the normal ($\beta^A$) gene.[2,3] Sickle cell anemia, which is found primarily in African populations, is caused by homozygosity for a unique DNA base-pair substitution ($\beta^S$) in the sixth codon of the gene.[4] β-Thalassemia is widespread in Mediterranean, Southeast Asian, African, and Indian populations and is due to a heterogeneous collection of mutations that seriously restricts or completely eliminates the production of β-globin protein. It is usually clinically manifested in homozygotes as a severe, transfusion-dependent hemolytic anemia (Cooley's anemia). Although at least 47 mutations of the β-globin gene that can lead to β-thalassemia have been characterized, a subset of common mutations is present in each ethnic group in which this disease is prevalent.[5-10] For example, four such mutations — the nonsense mutation in the 39th codon of the gene ($\beta^{39}$) and three RNA processing (splicing) mutations in the first intervening sequence at nucleotide positions 1, 6, and 110 ($\beta^{IVS1-1}$, $\beta^{IVS1-6}$, and $\beta^{IVS1-110}$, respectively) — together account for 79 percent of the β-thalassemia alleles present in Mediterranean populations[5] and 94 percent of the alleles in Sicilians.[11] The frequency, broad geographic distribution, and clinical severity of these hemoglobinopathies make testing for carriers and prenatal diagnosis a critical public health issue in these areas. Current hematologic tests are adequate for the screening of carriers, but the identification of specific mutations and prenatal diagnosis during the first trimester can be accomplished only with a DNA-based genetic test.

An important advance in the ability to detect these small genetic lesions directly was made several years ago, when a technique based on the differential hybridization of short, radioactively labeled oligonucleo-

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tide probes was introduced.[12-14] These synthetic DNA probes, typically 19 bases in length, are designed to span the region of the gene where the aberrant sequence is located. When used under appropriate conditions, such probes will anneal only to sequences with which they are perfectly matched; the mismatch of a single base pair is sufficiently destabilizing to prevent hybridization. Thus, two of these allele-specific oligonucleotide probes — one specific for the normal sequence and the other for its mutant counterpart — can directly determine the genotype of a DNA sample at that locus. However, because of the complexity of human genomic DNA and inefficiencies in hybridization that are inherent in short oligonucleotide probes, microgram quantities of sample DNA, gel electrophoresis, and highly radioactive DNA probes are required for this approach.

Recently, we described a modification of this method that uses an in vitro procedure to amplify DNA — the polymerase chain reaction — to amplify selectively a segment of the β-globin gene approximately $10^6$ times to facilitate the diagnosis of sickle cell anemia.[15] As a result of this substantial enrichment of the region of DNA of interest, a simplified "dot blot" detection format was possible that required only nanogram amounts of sample DNA and moderately radioactive oligonucleotide probes. Subsequent technological improvement of the amplification procedure, with the use of a thermostable DNA polymerase, greatly increased the specificity and yield of the reaction and made the process amenable to automation.[16] The reliance on radioactive probes, however, has limited the clinical applicability of this diagnostic test. In this report, we extend this general approach to include the analysis of the β-thalassemia alleles $\beta^{39}$, $\beta^{IVS1\text{-}1}$, $\beta^{IVS1\text{-}6}$, and $\beta^{IVS1\text{-}110}$, and introduce the use of nonradioactive, allele-specific oligonucleotide probes covalently attached to the enzyme horseradish peroxidase. These enzyme–oligonucleotide conjugate probes are readily indicated by a simple colorimetric reaction involving the enzymatic oxidation of a colorless soluble chromogen to a blue insoluble product.

## Methods

### Preparation of the Probes

The horseradish peroxidase–labeled oligodeoxyribonucleotide probes were prepared by covalently coupling oligomers containing a free sulfhydryl group at their 5′ ends with thiol-specific, maleimido-derivatized horseradish peroxidase (Chang C-A, et al.: unpublished data). In brief, the oligonucleotides were synthesized on an automated DNA synthesizer (Model 8700, Biosearch) with β-cyanoethyl *N,N*-diisopropyl phosphoramidite nucleosides (American Bionetics), according to protocols supplied by the manufacturer. A modified tetraethylene glycol, containing a triphenylmethyl mercaptan and a β-cyanoethyl *N,N*-diisopropyl phosphoramidite functional group, was employed to introduce the thiol moiety. As a nucleoside analogue, this reagent could be incorporated into the 5′ terminus of an oligonucleotide during the final round of synthesis; this is a variation of a previously described procedure.[17] The triphenylmethyl thio oligomers were purified by reversed-phase high-pressure liquid chromatography, and the triphenylmethyl group was removed by treatment with silver nitrate. The thiolated oligomers were then conjugated to horseradish peroxidase (Sigma) that had been modified by reaction with a maleimido-active ester. The resulting horseradish peroxidase–oligonucleotide was purified through high-pressure liquid chromatography by elution from ion-exchange column (DEAE) with a sodium chloride gradient. The ratio of horseradish peroxidase to oligonucleotide in the final product was measured by ultraviolet spectroscopy and determined to be essentially equimolar. The enzymatic activity of the horseradish peroxidase in the conjugate was assayed colorimetrically and found to be comparable to that of the native, unmodified enzyme. The conjugates are very stable and have been stored in 600 mM sodium chloride with 20 mM sodium phosphate buffer, pH 6.5, at 4°C for over one year with no marked loss of activity.

### Amplification of DNA

Samples of genomic DNA were amplified by using the *Thermus aquaticus (Taq)* heat-stable DNA polymerase as described previously.[16] Amplifications were performed with 0.25 to 0.5 μg of DNA in a 50-μl solution containing 50 mM potassium chloride, 10 mM TRIS, pH 8.4, 2.5 mM magnesium chloride, 1 μM each primer (KM29 and RS42), 200 μM each deoxyribonucleotide triphosphate (dATP, dCTP, TTP, and dGTP), 100 μg of gelatin per milliliter, and 2 units of *Taq* polymerase (Perkin Elmer-Cetus Instruments). The samples were overlaid with several drops (≈50 μl) of mineral oil to prevent condensation and were subjected to 30 cycles of amplification. The cycling reaction was performed in a programmable heat block (DNA Thermal Cycler, PECI) set to heat the samples from 72°C to 95°C over a 1-minute period (to denature the DNA), cool them to 55°C over 1 minute (to anneal the primers), heat them to 72°C in 30 seconds, and incubate them at that temperature for an additional 2 minutes (to extend the annealed primers). After the last cycle, the samples were incubated for an additional 5 minutes at 72°C to ensure that the final extension step was complete. By means of this protocol, 30 cycles of amplification could be accomplished in approximately 2.5 hours.

### Hybridization and Detection of the Probes

Five microliters of each sample of amplified DNA was mixed with 100 μl of 0.4 N sodium hydroxide and 25 mM EDTA and applied to nylon filter membranes (Genatrans-45, Plasco) as dot blots.[15] Replicate filters were prepared as necessary. The DNA was fixed to the nylon by ultraviolet irradiation of the damp filters for five minutes (Model TM-36 Transilluminator, UV Products).[18] The filters were hybridized with 1 to 2 pmol of horseradish peroxidase–labeled, allele-specific oligonucleotide probe in 4 ml of 5× SSPE, 5× Denhardt's solution, and 0.5 percent Triton X-100 for 30 minutes at 42°C, then quickly rinsed three times in 100 ml of 2× SSPE and 0.1 percent Triton X-100 at room temperature to remove excess probe. (SSPE denotes 0.015 M sodium citrate, 0.012 M sodium chloride, 0.013 M potassium phosphate, 0.001 M EDTA [pH 7.2].) A final high-stringency wash was performed in the wash buffer at 42°C for five minutes. Detection of the annealed probe was carried out essentially as described by Sheldon et al.[19] for the detection of streptavidin–horseradish peroxidase. The hybridized filters were first incubated for five minutes at room temperature in 100 ml of phosphate-buffered saline containing 100 mM sodium chloride, 1 M urea, 5 percent Triton X-100, and 1 percent dextran sulfate, then equilibrated in 100 mM sodium citrate, pH 5.0, containing 0.1 mg of 3,3′,5,5′-tetramethylbenzidine per milliliter (Fluka). Color development was accomplished by transferring the filters to sodium citrate–tetramethylbenzidine buffer containing 0.0015 percent hydrogen peroxide, and incubating them for 10 to 20 minutes at room temperature. The filters were extensively washed in deionized water and, for maximum stability, stored moist in heat-sealed plastic pouches. Photographs were taken for permanent records.

## Results

The portion of the human β-globin gene to be amplified by the polymerase chain reaction is shown in Figure 1. The two amplification primers, KM29 and RS42, flank and define a 536-base-pair target segment

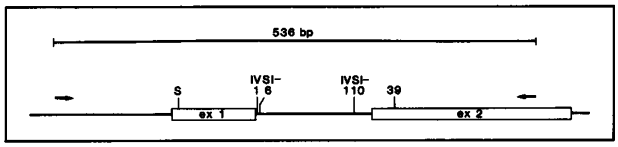


Figure 1. Region of the β-Globin Gene to Be Amplified and Locations of the Mutations for Sickle Cell Anemia and β-Thalassemia.
The boxes represent translated portions of the gene that code for β-globin protein (exons 1 and 2), and the horizontal lines adjacent to the boxes represent untranslated portions of the gene that do not code for protein (5′ untranslated, first and second intervening sequences). The arrows indicate the sites where the primers, KM29 and RS42, anneal to the gene and the 536-base-pair (bp) segment that they define as the target of amplification. Tick marks indicate the locations of the mutations: $\beta^S$, $\beta^{IVS1-1}$, $\beta^{IVS1-6}$, $\beta^{IVS1-110}$, and $\beta^{39}$.

of the gene, from the 5′ untranslated region to the latter part of the second exon, where the sickle cell mutation and the majority of the known β-thalassemia mutations are found.[2,3] Amplification according to this procedure is achieved by repeated cycles of DNA denaturation, annealing of the primers to their complementary sequences, and extension of the annealed primers by DNA polymerase. Initially, these primers hybridize to opposite strands of the denatured β-globin gene and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of the β-globin DNA segment. Because the extension products are themselves complementary to and capable of binding primers, each successive cycle continues essentially to double the amount of DNA synthesized in the previous cycle. After 30 such repetitions, the targeted β-globin fragment can be amplified $10^7$-fold.[16] The sequences of the amplification primers and the 15-base oligonucleotide probes specific for the $\beta^S$, $\beta^{39}$, $\beta^{IVS1-1}$, $\beta^{IVS1-6}$, and $\beta^{IVS1-110}$ normal and mutant alleles are listed in Table 1.

The specificity of the horseradish peroxidase–labeled, allele-specific oligonucleotide probes was assessed in two groups of previously characterized DNA samples containing the $\beta^{39}$ or $\beta^{IVS1-110}$ mutations. After amplification, portions of the reaction products were spotted onto nylon membranes. Two replicate filters were prepared for each sample set, and each was hybridized with one of the enzyme-labeled oligonucleotide probes under stringent conditions that permitted discrimination between alleles. Color development of the filters revealed the presence of the hybridized probe as a blue precipitate (Fig. 2). The β-globin genotype of each sample of amplified DNA was readily determined by observing the presence or absence of a signal for each probe. For example, sample 2 was interpreted as homozygous for the $\beta^{39}$ sequence since the probe specific for the $\beta^{39}$ mutation, HRP-15M39, hybridized to the sample, whereas the probe for the normal sequence, HRP-15N39, did not. Similarly, sample 12 was judged to be

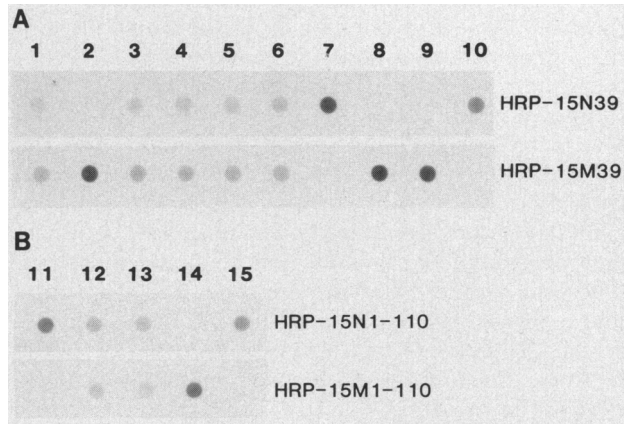


Figure 2. Specificity of the Horseradish Peroxidase–Oligonucleotide Probes for β-Thalassemia Alleles on Amplified DNA.
The amplified DNA (1-15) was spotted onto duplicate nylon membranes and hybridized with the indicated probes under stringent conditions as described in the text. Part A shows the analysis of 10 selected DNA samples with oligonucleotide probes specific for the normal (HRP-15N39) and mutant (HRP-15M39) sequences at the 39th codon of the gene (HRP denotes horseradish peroxidase). Samples 7 and 10 are homozygous for the normal gene ($\beta^A\beta^A$); samples 1 and 3 through 6 are heterozygous, possessing one copy of each allele ($\beta^A\beta^{39}$); samples 2, 8, and 9 are homozygous for the mutant allele ($\beta^{39}\beta^{39}$). Part B shows the results of a similar analysis on five additional samples, with probes for the normal (HRP-15N1-110) and mutant (HRP-15M1-110) sequences at nucleotide position 110 in the first intervening sequence. Samples 11 and 15 are homozygous for the normal allele ($\beta^A\beta^A$); samples 12 and 13 are heterozygous ($\beta^A\beta^{IVS1-110}$); sample 14 is homozygous for the mutant gene ($\beta^{IVS1-110}\beta^{IVS1-110}$).

Table 1. Sequence of Oligonucleotide Primers and Probes.

| NAME | FUNCTION | SEQUENCE* |
|---|---|---|
| KM29 | β-Globin primer | GGTTGGCCAATCTACTCCCAGG |
| RS42 | β-Globin primer | GCTCACTCAGTGTGGCAAAG |
| HRP-15A | Normal $\beta^6$ probe | HRP-CCTGAGGAGAAGTCT |
| HRP-15S | Mutant $\beta^6(\beta^S)$ probe | HRP-CCTGTGGAGAAGTCT |
| HRP-15N39 | Normal $\beta^{39}$ probe | HRP-TTGGACCCAGAGGTT |
| HRP-15M39 | Mutant $\beta^{39}$ probe | HRP-TTGGACCTAGAGGTT |
| HRP-15N1,6† | Normal $\beta^{IVS1-1,6}$ probe | HRP-GCAGGTTGGTATCAA |
| HRP-15M1 | Mutant $\beta^{IVS1-1}$ probe | HRP-GCAGATTGGTATCAA |
| HRP-15M6 | Mutant $\beta^{IVS1-6}$ probe | HRP-GCAGGTTGGCATCAA |
| HRP-15N110 | Normal $\beta^{IVS1-110}$ probe | HRP-CTGCCTATTGGTCTA |
| HRP-15M110 | Mutant $\beta^{IVS1-110}$ probe | HRP-CTGCCTATTAGTCTA |

*The β-globin mutations are underlined.

†Because there are only four nucleotides between the $\beta^{IVS1-1}$ and $\beta^{IVS1-6}$ mutations, a single oligonucleotide probe was able to detect the normal sequence at either position.

heterozygous for the normal and mutant $\beta^{IVS1-110}$ alleles since signals for both probes, HRP-15N1-110 and HRP-15M1-110, were visible. The intensity of the signals obtained with these nonradioactive probes was 1 to 10 percent of the signal intensity obtained with the equivalent 15-base, $^{32}$P-labeled allele-specific oligonucleotide probes (specific activity, 4 μCi per picomole) after 15 minutes' autoradiographic exposure (data not shown).

Retrospective analysis was performed on two pregnancies at risk for β-thalassemia (families I and II), for which prenatal diagnosis had been requested. Fetal DNA was isolated from amniocytes, and parental DNA from peripheral-blood lymphocytes.[20] The diagnoses were originally determined by means of radio-

labeled allele-specific oligonucleotide probes; both parents of family I were carriers of the $\beta^{39}$ mutation, and the fetus was homozygous for the normal ($\beta^{A}$) gene. The mother of family II was a carrier of the $\beta^{39}$ allele as well, but the father carried the $\beta^{IVS1-1}$ mutation. The fetus of family II was also homozygous for the normal gene. The results of the analysis of these amplified DNA samples with the $\beta^{39}$ and $\beta^{IVS1-1}$ horseradish peroxidase–oligonucleotide probes were consistent with the initial conclusions (Fig. 3).

The diagnostic capability of the nonradioactive probes was demonstrated in assorted clinical DNA samples containing the $\beta^{A}$, $\beta^{39}$, $\beta^{IVS1-1}$, $\beta^{IVS1-6}$, or $\beta^{IVS1-110}$ alleles. Nine samples were arranged into three family sets to simulate cases submitted for prenatal diagnosis, then coded so that the $\beta$-globin genotypes were not known at the time of analysis. Each sample was tested for the presence of each of the four $\beta$-thalassemia mutations. After amplification, the samples were applied to eight replicate filters, and each filter was hybridized with one of the eight enzyme-labeled probes (Fig. 4). In family III, both parents were predicted to be carriers of the $\beta^{39}$ mutation. The fetus inherited both mutant genes from its parents and, being homozygous for $\beta^{39}$, would have $\beta$-thalassemia. The father of family IV was also a carrier of the $\beta^{39}$ mutation, and the mother was genotyped as heterozygous for the $\beta^{IVS1-110}$ allele. The fetus in this family inherited one copy of the normal $\beta$-globin gene from its father and one copy of $\beta^{IVS1-110}$ from its mother, and was diagnosed as a carrier of the $\beta$-thalassemia gene. The parents of family V also had different

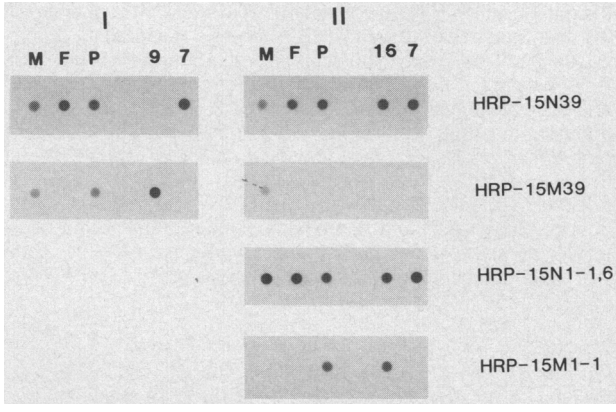


Figure 3. Results of a Retrospective Analysis of Two Pregnancies at Risk for $\beta$-Thalassemia.

Both families (I and II) were tested for the $\beta^{39}$ mutation by means of the HRP-15N39 and HRP-15M39 probes. Family II was also examined for the $\beta^{IVS1-1}$ mutation (at nucleotide position 1 in the first intervening sequence) with the HRP-15N1-1,6 and HRP-15M1-1 probes. The confirmed $\beta$-globin genotypes of the members of family I were $\beta^{A}\beta^{39}$ for both the maternal (M) and paternal (P) samples, and $\beta^{A}\beta^{A}$ for the fetal (F) sample. Control samples 9 and 7 are described in Figure 2. The genotypes of family II were $\beta^{A}\beta^{39}$ for the maternal sample, $\beta^{A}\beta^{A}$ for the fetal sample, and $\beta^{A}\beta^{IVS1-1}$ for the paternal sample. Control sample 16 was obtained from a subject known to be heterozygous for $\beta^{A}\beta^{IVS1-1}$

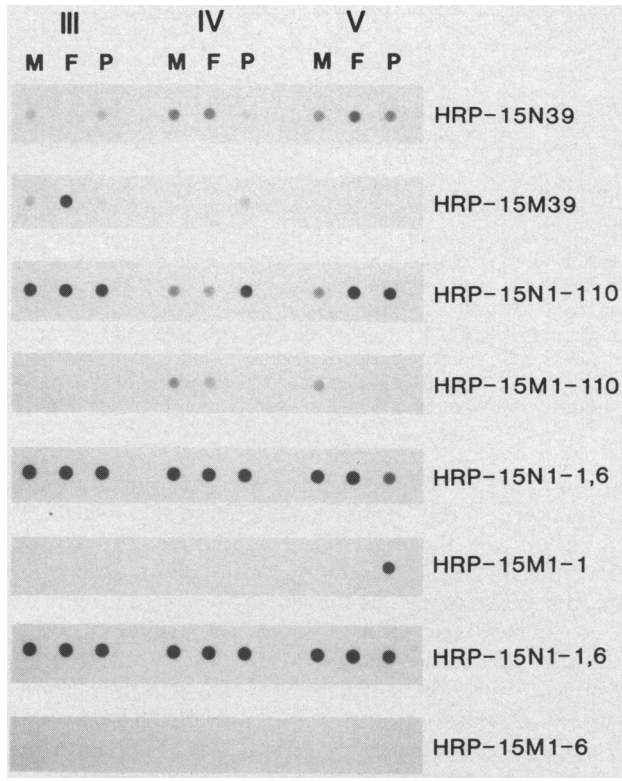


Figure 4. Diagnosis of $\beta$-Thalassemia in Three Family Sets.

The families (III through V) were tested with each of the probes described in Figures 1 and 2, as well as a probe for the $\beta^{IVS1-6}$ mutation (at nucleotide position 6 in the first intervening sequence) with the probe HRP-15M1-6. The confirmed $\beta$-globin genotype of the members of family III was $\beta^{A}\beta^{39}$ for maternal DNA, $\beta^{39}\beta^{39}$ for fetal DNA, and $\beta^{A}\beta^{39}$ for paternal DNA; in family IV, $\beta^{A}\beta^{IVS1-110}$, for maternal DNA, $\beta^{A}\beta^{IVS1-110}$ for fetal DNA, and $\beta^{A}\beta^{39}$ for paternal DNA; and in family V, $\beta^{A}\beta^{IVS1-110}$ for maternal DNA, $\beta^{A}\beta^{A}$ for fetal DNA, and $\beta^{A}\beta^{IVS1-1}$ for paternal DNA.

$\beta$-thalassemia genes; the father was a carrier of $\beta^{IVS1-1}$, and the mother was a carrier of $\beta^{IVS1-110}$. In this case, however, the fetus inherited only the normal genes from its parents. Decoding showed that all samples were correctly identified.

In the final example, retrospective analysis was performed on a pregnancy in which dizygotic twins were at risk for sickle cell anemia. Both fetal sacs underwent sampling by amniocentesis, and DNA was prepared from the collected amniocytes. DNA was obtained from peripheral-blood lymphocytes of each parent. After amplification of the parental and fetal DNA samples, analysis with the probes for the sickle cell mutation confirmed that both parents were obligate carriers. One fetus was shown to be homozygous for the normal allele, and the other to be a heterozygous carrier, like its parents (Fig. 5).

## Discussion

The hereditary hemoglobinopathies are among the best-characterized human genetic diseases. Consequently, they have served as a model for the development of several DNA-based strategies for diagnosis.

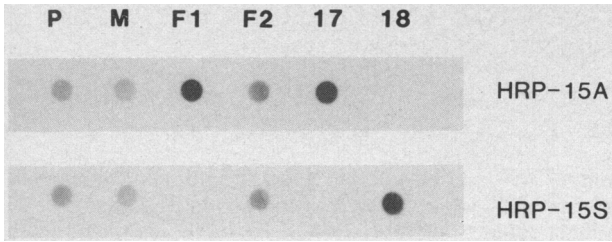


Figure 5. Results of a Retrospective Analysis of a Dizygotic Pregnancy at Risk for Sickle Cell Anemia.

The samples were tested with the probe specific for the normal sequence at codon 6 (HRP-15A) and the probe for the sickle cell mutation (HRP-15S). The confirmed β-globin genotypes of the amplified DNA samples in this family were $\beta^A\beta^S$ for both maternal and paternal samples, $\beta^A\beta^A$ for the sample from twin 1 (F1), and $\beta^A\beta^S$ for the sample from twin 2 (F2). Control sample 17 was obtained from a subject known to be homozygous for $\beta^A\beta^A$ who was normal, and control sample 18 from a subject known to be homozygous for $\beta^S\beta^S$ who had sickle cell anemia.

One of the most versatile of these methods exploits the differential hybridization of radioactively labeled, allele-specific oligonucleotide probes to detect genetic mutations directly. Although it is a powerful diagnostic tool, this technique has had limited clinical application because of its relative insensitivity and procedural complexity. In this report, we have described an approach to the diagnosis of sickle cell anemia and β-thalassemia that uses the polymerase chain reaction to increase the amount of β-globin target sequence before genetic analysis with nonradioactive, enzyme-labeled oligonucleotide probes in a simple dot-blot format. These horseradish peroxidase–oligonucleotide probes are accurate and convenient to use and remain stable for at least one year. Moreover, because extensive purification methods are not required to obtain amplifiable DNA,[15,21] the entire analysis can be accomplished in six hours or less: three hours for sample preparation and amplification, one hour for filter preparation, and two hours for probe hybridization and detection. The generality and sensitivity of this procedure have been illustrated by the genetic typing of the polymorphic HLA-DQα locus with a panel of enzyme-labeled oligonucleotide probes on DNA amplified from single hairs.[22]

Three recently described procedures for genetic analysis[21,23,24] also incorporate the amplification reaction, but involve restriction-enzyme cleavage of the amplified product in the diagnosis of hemophilia A, β-thalassemia, and sickle cell anemia. Although a simple and elegant approach, restriction-enzyme cleavage is limited to linkage analysis of polymorphic restriction sites and to situations in which the mutation fortuitously creates or disrupts a restriction site. It also requires that the amplification reaction produce a unique product fragment. Genetic analysis with nonradioactive allele-specific oligonucleotide probes should prove to be a more general solution. Indeed, the broad potential of DNA amplification followed by analysis with a nonisotopic oligonucleotide probe lies not only in the diagnosis of the hereditary hemoglobinopathies as reported here, but in its general applicability to testing for carriers and prenatal diagnosis in many other genetic diseases.

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