Rapid Screening of Multiple β-Globin Gene Mutations by Real-Time PCR on the LightCycler: Application to Carrier Screening and Prenatal Diagnosis of Thalassemia Syndromes

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Background: Hemoglobinopathies are priority genetic diseases for prevention programs. Rapid genotype characterization is fundamental in the diagnostic laboratory, especially when offering prenatal diagnosis for carrier couples.

Methods: As a model, we designed a protocol based on the LightCycler™ technology to screen for a spectrum of β-globin gene mutations in the Greek population. Design was facilitated by dual fluorochrome detection and close proximity of many mutations. Three probe sets were capable of screening 95% of β-globin gene mutations in the Greek population, including IVSII-745C→G, HbS, Cd5-CT, Cd6-A, Cd8-AA, IVSII-1G→A, IVSII-5G→A, IVSII-6T→C, IVSII-110G→A, and Cd39 C→T.

Results: The protocol, standardized by analysis of 100 β-thalassemia heterozygotes with known mutations, was 100% reliable in distinguishing wild-type from mutant alleles. Subsequent screening of 100 Greek β-thalassemia heterozygotes with unknown mutations found 96 of 100 samples heterozygous for 1 of the 10 mutations, although melting curves were indistinguishable for mutations HbS/Cd6 and IVSII-5/IVSII-1, indicating a need of alternative methods for definitive diagnosis. One sample demonstrating a unique melting curve was characterized by sequencing as Cd8/9+G. Three samples carried mutations outside the gene region covered by the probes. The protocol was 100% accurate in 25 prenatal diagnosis samples, with 14 different genotype combinations diagnosed. The protocol was also flexible, detecting five β-globin gene mutations from other population groups (IVSII-1G→T, IVSII-5G→C, IVSII-116T→G, Cd37 TGG→TGA, and Cd41/42 TCTT).

Conclusions: The described LightCycler system protocol can rapidly screen for many β-globin gene mutations. It is appropriate for use in many populations for directing definitive mutation diagnosis and is suited for rapid prenatal diagnosis with low cost per assay.

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The inherited disorders of hemoglobin (Hb)3 synthesis constitute the most common monogenic diseases worldwide and include the thalassemia syndromes and structural Hb variants such as HbS, HbE, HbC, HbDpuntjab and HbOArab. The clinical severity of thalassemia major and the sickle cell syndromes makes them priority genetic diseases for prevention programs involving population screening for heterozygotes and for optional prenatal diagnosis for carrier couples.

The ability to perform rapid DNA analysis for genotype characterization has become an increasingly important requirement for the clinical diagnostic laboratory. Currently, a wide variety of methods exist for detecting point mutations in a DNA molecule. Classic methods include oligonucleotide hybridization, endonuclease restriction analysis of PCR products, the Amplification Refractory Mutation System (ARMS), denaturing gradient gel electrophoresis, and direct sequencing. All of the above methods require several hours, and sometimes

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3 Nonstandard abbreviations: Hb, hemoglobin; ARMS, Amplification Refractory Mutation System; and T_m, melting temperature.
days, for complete diagnosis (1); consequently, there is a need for more rapid, high-throughput assays.

Recently, systems have been developed that integrate microvolume rapid-cycle PCR with fluorometry, offering real-time fluorescent monitoring of the amplification reaction for quantitative PCR and/or characterization of PCR products for rapid genotyping without the need for post-PCR sample manipulation (2, 3). The LightCycler™ (Roche Molecular Biochemicals) is one such system. The detection of potential sequence differences (usually single-nucleotide polymorphisms) for genotyping applications involves the use of two fluorescently labeled probes that hybridize to adjacent internal sequences within target amplified DNA, one of which covers the region expected to contain the mutation(s). Close proximity of annealed probes facilitates fluorescence resonance energy transfer between them. Probes are designed to have different melting temperatures (Tm,s), such that the probe with the lower Tm lies over the mutation site(s). Monitoring of the emitted fluorescent signals as the temperature increases detects loss of fluorescence as the probe with the lower Tm melts off the template. A single base mismatch under this probe produces a Tm shift of 5–10°C, allowing easy distinction between wild-type and mutant alleles. The ability to detect base mismatches under the probe with the lower Tm (mutation detection probe) and the use of two, different-colored probes allows more than one mutation to be screened in a single PCR reaction. Because of its low costs, reproducibility, and ease of handling, the assay is potentially suited for the routine clinical laboratory.

The use of this system for fast genotyping of three common β-hemoglobin variants has been demonstrated previously (4). In this study we report the standardization of a real-time detection method for most of the common β-thalassemia mutations throughout the world (5, 6) along with the Hbs mutation, using the Greek population as a model. In Greece, up to 10% of the population carry β-thalassemia or related hemoglobin variants, and ∼30 pathologic point mutations in the β-globin gene (5–8) have been observed, with 9 accounting for ∼95% of the affected alleles (5, 6). The method was rapid and accurate with high throughput for screening β-globin gene mutations in β-thalassemia heterozygotes. More importantly, for prenatal diagnosis, the LightCycler method was faster than the routine methodology (1) and equally accurate, as demonstrated with 25 prenatal diagnosis samples that included 14 different genotype combinations.

Materials and Methods

Materials

The LightCycler PCR method was standardized with use of DNA samples of Greek origin from 100 β-thalassemia heterozygotes with β-thalassemia mutations characterized previously by direct mutation assays (1, 8). The method was then used to analyze DNA from 100 samples (Greek origin) with hematologic findings consistent with heterozygous β-thalassemia, from 25 samples referred for prenatal diagnosis of possible β-thalassemia major or sickle cell syndromes (17 chorionic villi samples and 8 amniotic fluid samples), and from 5 DNA samples (from various laboratory DNA banks) from other population groups with mutations different from those found in the Greek population.

Isolation of Genomic DNA

Genomic DNA was isolated with use of the QIAamp DNA Blood Mini Kit (Qiagen) from (a) 200 μL of EDTA-anticoagulated whole blood for the thalassemia heterozygotes, (b) 200 μL of amniotic cell suspension in a solution of 9 g/L NaCl (concentrated after centrifugation of 10–15 mL of amniotic fluid samples at 3040g) for the prenatal samples, and (c) two or three chorionic villi in 200 μL of a solution containing 9 g/L NaCl, selected after viewing under the microscope, for the samples from laboratory DNA banks outside of Greece.

Design of PCR Primers and Mutation Detection Probes

PCR and mutation detection by melting curve analysis were performed on the LightCycler, which can simultaneously measure emitted signals from two different fluorophores. PCR primers (forward, 5'-gtc atc act tag acc tca-3'; reverse, 5'-cag gca gcc ctc gta g-3') were designed with the aid of the computer software Amplify, Ver. 2.0, 1992–1995 (Bill Engels) to amplify a 587-bp region of the β-globin gene surrounding the most common β-thalassemia mutations in the Greek population (and most of the common mutations in all world populations), along with the Hbs mutation (Fig. 1) (5, 6).

The clustering of many mutations within small distances facilitated the design of just three combinations (or sets) of probes for mutation detection. One mutation relatively common in the Greek population (IVSII-745; 7% of β-thalassemia mutations) (5) is not located within the amplified 587-bp 5' region of the gene, but this mutation has been observed in cis with a polymorphic base change (+20C→T) in the 5' untranslated region in more than 200 samples with IVSII-745; we thus chose to design a detection probe specific for the linked polymorphic change (+20C→T) that would allow indirect screening for the mutation IVSII-745 (unpublished data). The acceptor mutation detection probes were designed by TIB MOLBIOL.

Two of the probe combinations (sets A and C) included two acceptor (mutation detection) probes with one central donor probe (Table 1 and Fig. 1), which would enable the use of one or both acceptor probes of the set according to the needs of any genotyping assay. The acceptor probes in sets A and C were labeled with different acceptor fluorophores (LightCycler Red 640 or LightCycler Red 705), and the central donor probe, which was designed to span the distance between the two acceptor probes, was labeled with a fluorescein molecule at both the 5' and 3' ends. Set B was designed to screen for three neighboring mutations with use of a single mutation detection (acceptor) probe,
labeled with LightCycler Red 705, in combination with a donor probe that was labeled with fluorescein only at the end adjacent to the acceptor probe (Table 1 and Fig. 1). In all sets, the mutation-screening (acceptor) probes were designed to have a lower \( T_m \) relative to the donor probes, thereby ensuring that the fluorescent signal generated during the melting curve analysis was produced only by the mutation probe, and all were complementary for the wild-type sequence.

PCR amplification and mutation detection

PCR amplification reactions were carried out in LightCycler glass capillary tubes (Roche) with \( 50 \) ng of genomic DNA in a total reaction volume of \( 20 \mu L \) containing the ready-to-use reaction mixture provided by the manufacturer (LightCycler DNA Master Hybridization Probes) with \( 4 \) mM \( Mg^{2+} \), \( 0.5 \mu M \) each PCR primer, and \( 0.15 \mu M \) each fluorescent probe. The PCR amplification included a first denaturation step of 30 s at 95 °C, followed by 35 cycles of 95 °C for 3 s, 58 °C for 5 s, and 72 °C for 20 s with a temperature ramp of 20 °C/s. During the PCR, emitted fluorescence was measured at the end of the annealing step of each amplification cycle to monitor amplification.

Genotypes were determined by melting curve analysis of probes immediately after the amplification step. This involved a momentary increase in temperature to 95 °C, cooling to 45 °C for 2 min to achieve maximum probe
hybridization, and then heating to 85 °C at a rate of 0.4 °C/s, during which time the melting curve was recorded. Emitted fluorescence was measured continuously [by both channels F2 (640 nm) and F3 (705 nm) if necessary] to monitor the dissociation of the fluorophore-labeled detection probes from the complementary single-stranded DNA (F/T). The computer software automatically converted and displayed the first negative derivative of the F/T ratio vs temperature (−dF/dT vs T), and the resulting melting peaks allowed easy differentiation between wild-type and mutant alleles.

**Results**

The LightCycler method was standardized by analyzing DNA from 100 β-thalassemia heterozygotes with nine known β-thalassemia mutations or HbS (10 samples each for HbS, IVS-I-5, IVS-I-6, and IVS-I-745; 5 samples each for CdS, Cd6, and Cd8; 15 samples each for IVS-I-110, Cd39, and IVS-I-1). A separate experiment was set up for each mutation with the appropriate pair of acceptor/donor probes. Each PCR reaction included heterozygous and homozygous (when available) samples for the mutation under evaluation, five controls negative for the mutation, and a DNA-free blank to monitor the absence of contamination. In all sets of experiments for each mutation, the melting curves with the detection probe distinguished the wild-type from mutant alleles, allowing assignment of wild-type, heterozygous, or homozygous status to each sample as expected. No false-positive or false-negative results or contamination was observed in any case.

To screen the unknown mutations in 100 β-thalassemia heterozygotes, who had been diagnosed hematologically, we applied a strategy based on the distribution of the mutations in the Greek population. Using a single genotyping assay with set C hybridization probes (Table 1, Fig. 1, and Fig. 2, D and E), we first investigated mutations IVS-I-110 and Cd39 (expected to account for ~60% of β-thalassemia alleles in Greece). Samples that were negative were then investigated using probe set B (Table 1, Fig. 1, Fig. 2C, and Fig. 3B) for the mutations IVS-I-1, IVS-I-5, and IVS-I-6, which account for an additional 20% of β-thalassemia alleles. Finally, we used set A to investigate the +20C→T polymorphism (linked to IVS-I-745 mutation) and HbS, Cd5, Cd6, and Cd8 mutations (Table 1; Fig. 1, Fig. 2, A and B; and Fig. 3A). Melting curves in 97 of the samples indicated heterozygosity for a mutation within the β-globin gene regions covered by the mutation detection probes. On the other hand, we noticed that some mutations, including HbS with Cd6 (Fig. 3A) or IVS-I-5 with IVS-I-1 (Fig. 3B), had identical melting curves, which were, however, distinct from the wild type. In such cases, the IVS-I-1 mutation was identified by use of ARMS (9), and the IVS-I-5 mutation was confirmed by digestion with EcoRV (1). The HbS mutation can be distinguished from Cd6 on the basis of hematologic findings with direct mutation identification based on ARMS PCR (9).

Through comparisons with controls analyzed simultaneously in each assay, we characterized 96 of the 100 samples as heterozygous for 1 of 10 mutations, including 21 with HbS or Cd6 (19 with HbS and 2 with Cd6 as determined by ARMS), 1 with Cd5, 3 with Cd8, 37 with IVS-I-110, 11 with Cd39, 5 with IVS-I-6, 3 with the +20
polymorphism (linked to IVSII-745), and 15 with IVSI-1 or IVSI-5 (13 with IVSI-1 and 2 with IVSI-5 as determined by ARMS and EcoRV digestion). In one sample, we observed a different melting curve, very close to that for the wild type, for the mutation detection probe Ac Cd5.6.8 (Fig. 2B and Fig. 3Aiv); after sequencing, this sample was found to have the Cd8/9+G mutation, which is rare in the Greek population. All genotype results were confirmed by routine methodologies, including denaturing gradient gel electrophoresis in combination with ARMS PCR, endonuclease restriction analysis, or direct sequencing. The remaining three \( \beta \)-thalassemia heterozygotes were found to carry mutations outside the region of the gene included in the assay: \(-87C\rightarrow G, \) poly A (AATAAA \( \rightarrow \) AATGAA, where the substituted base is shown italics), or IVSII-1 G\( \rightarrow \) A. 

The protocol was also used to investigate the genotypes in 25 prenatal diagnosis samples at risk for \( \beta \)-thalassemia major or sickle cell disease, based on previously identified parental mutations in each case. Fourteen different genotypes were identified: IVSI-110/IVSI-110, IVSI-110/IVSI-1, IVSI-110/IVSI-6, IVSI-110/IVSI-5, IVSI-110/Cd39, IVSI-110/HbS, Cd39/Cd39, Cd39/HbS, Cd39/IVSI-5, IVSI-745/IVSI-6, IVSI-745/HbS, IVSI-1/IVSI-5, IVSI-5/IVSI-6, and HbS/HbS. All results were in complete concordance with the genotypes obtained in parallel by routine methodologies as above (1), identifying 9 wild-type, 10 heterozygous, and 6 \( \beta \)-thalassemia major fetuses. The genotype results obtained with the LightCycler protocol were completed within a single working day, whereas those obtained with routine methods required 2–3 days.

Finally, the protocol was able to distinguish the mutant alleles in five DNA samples from other population groups with mutations (previously characterized) not found in the Greek population but within the gene regions covered by the mutation detection probes designed in this protocol (IVSI-1G\( \rightarrow \) T, IVSI-5G\( \rightarrow \) C, IVSI-116T\( \rightarrow \) G, Cd37 TGG\( \rightarrow \) TGA, and Cd41/42 -TCTT). All the above mutations produced melting curves distinct from the wild type. Specifically, the melting curves for the four variant alleles associated with base changes in IVSI-1 and IVSI-5...
were all identical. The melting curves for Cd37 and Cd41/42 were distinct from that for Cd39, but IVSI-116 and IVSI-110 had identical melting curves (data not shown).

**Discussion**

The β-globin gene is a relatively small gene (<2000 bp) located in the short arm of chromosome 11 (10). Although >180 causative mutations have been reported for β-thalassemia syndromes, the spectrum of mutations and their frequencies in most populations usually consist of a limited number of common mutations and a slightly larger number of rare mutations (11). Mutation characterization in carriers is a prerequisite when offering prenatal diagnosis to couples at risk for having an affected child and frequently needs to be achieved as quickly as possible, especially when at-risk couples present after establishment of a pregnancy. With the aim of rapid DNA analysis, real-time PCR has become an important tool in both research and routine clinical diagnostics (2, 3, 12–15), and use of the LightCycler facilitates simultaneous multiple mutation detection through the flexible design of detection probes. Greece is representative of a population with a heterogeneous spectrum of β-thalassemia muta-
tions and was considered an appropriate model for developing the described protocol. In Greece, ~30 mutations have been observed, with 9 accounting for ~95% of β-thalassemia alleles, most of which are situated in the first half of the gene in close proximity (5, 7, 8) (Fig. 1).

In this study, we designed a triplet reaction protocol to screen for multiple β-globin gene mutations, with use of five mutation probes, for the identification of at least 10 β-thalassemia mutations known in Greece as well as HbS.

An analogous LightCycler method has been described for genotyping samples with HbS and HbC. It used a detection probe complementary to the wild-type sequence. This approach was greatly facilitated by the close proximity of many β-thalassemia mutations known in Greece as well as HbS.

The protocol was standardized by analysis of β-thalassemia heterozygotes representing 10 different β-gene mutations found in Greece (Fig. 1 and Table 1) and demonstrated 100% reliability in distinguishing wild-type vs mutant alleles. The subsequent application of the protocol to screen samples from Greek β-thalassemia heterozygotes with unknown mutations allowed evaluation of the method and highlighted several points. The first point is that all samples with mutations within the gene regions covered by the LightCycler detection probes were positively detected as having a variant allele (97 of 100 Greek samples), which allowed all mutations to be distinguished reliably from wild-type alleles although mutations with very similar shifts in $T_m$ could not be differentiated from one another. Identification of a single sample with a β-thalassemia mutation (CD8/9+G) that is rare in the Greek population indicates that the system can also detect rare or novel mutations. The second point is that the probe sets were equally reliable when either single or double detection probes were used (as appropriate). Furthermore, the design of the probe set combinations enabled stepwise exclusion/detection of potential mutations, covering initially 60% (with set C), followed by evaluation of an additional 20% (with set B). Thus, based on the distribution and frequency of β-thalassemia mutations in the Greek population, only 20% of samples required the use of all three primer sets to assay for >95% of potential β-thalassemia mutations, minimizing the cost and time of the test for most samples. Overall, the LightCycler system provided a rapid screening protocol to locate the mutation, guiding the selection of the mutation-specific assay for definitive diagnosis.

Once the reliability and suitability of the method had been established for the Greek population, we then evaluated the suitability of the method for other populations and found that, according to reported mutation spectra and frequencies (5, 6), it was similarly useful for all populations of the Mediterranean region. In addition, we noted that other mutations common in other populations, such as the IVSI-1G→T, IVSI-5G→C, or Cd41/42→TCTT mutations common in Asia, were located under the mutation detection probes designed for the Greek population. The testing of appropriate samples demonstrated that the protocol is applicable for screening ≥75% of the common β-thalassemia mutations found on the Indian subcontinent and in Asia as well as in many populations of the Middle East (5, 6).

The LightCycler method was most advantageous when applied for prenatal diagnosis. Once the parental mutations are known, the method is very fast, requiring <3 h for completion, including DNA extraction from the fetal sample. Compared with current methods, this is very beneficial for the couple and the pregnancy. Furthermore, application of the described “Greek” LightCycler protocol in 25 prenatal samples for diagnosis for β-thalassemia and sickle cell syndromes demonstrated that 12 of the 14 genotype combinations among the 25 cases required only one PCR reaction, minimizing both the time and cost of prenatal diagnosis. Additionally, we observed that in cases in which the two parental mutations differed but were under the same detection probe, the similarity in $T_m$ did not produce ambivalent results because the melting curve of a wild-type allele was always distinct when present.

Overall, the proposed method is reliable, accurate, and rapid. It is useful for screening samples to direct definitive mutation diagnosis, as is necessary for at-risk couples before they proceed to prenatal diagnosis. It is especially suited for prenatal diagnosis because it is extremely rapid. It is also advantageous in terms of safety compared with conventional methods for mutation diagnosis because it avoids the need to handle hazardous chemicals (e.g., acrylamide or ethidium bromide). Finally, excluding the cost for the initial purchase of the LightCycler, the cost per assay is low.
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References


