an increased plasma 5-HIAA concentration in a carcinoid patient with atypical facial flush.

The application of the present assay allowed the measurement of very low concentration of plasma 5-HT and 5-HIAA. Plasma 5-HT may serve as a biomarker for patients with cancer, especially for intestinal cancers in the present study. Follow-up examinations and further investigations of the plasma 5-HT concentrations of those patients with intestinal cancers after dissection surgery are warranted.

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References


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Melting Temperature Assay for a UGT1A Gene Variant in Gilbert Syndrome, Nicola Marziliano,1* Elisabetta Pelo,2 Barbara Minuti,2 Ilaria Passerini,2 Francesca Torricelli,2 and Luigi Da Prato1 (1 Sequence Detection System/Genetic Analysis Core Laboratory, PE Europe BV, Via Tiepolo 18, 20052 Monza, Italy; 2 Cytogenetics and Genetic Unit, Florence Hospital “Careggi”, 50122 Florence, Italy; * author for correspondence: fax 39-039-8389497, e-mail nicola.marziliano@eur.pebio.com)

For detection of nucleotide polymorphisms or microinsertions/deletions, an optimal genotyping method would be rapid and economical. Newer, homogeneous, and automated genotyping methods with fluorescent oligonucleotide probes (1–3) can be expensive, and their use is difficult to optimize. Here we demonstrate the application of melting temperature (Tm) analysis (4) in a study of the UGT1A gene in a family with Gilbert syndrome.

Both amplification and Tm analysis are performed on the same fluorescence-detection thermocycler, the GeneAmp 5700 Sequence Detection System (PE Biosystems). The single-tube assay uses a specific primer set, with a high annealing temperature, and a fluorescent dye that can detect double-stranded DNA product. Because the temperature at which double-stranded templates dissociate depends largely on product length, GC content, and sequence structure, the PCR product has a distinct Tm depending on base substitutions, single point mutations, or insertions/deletions.

Soon after the last amplification cycle, the reaction is slowly cooled by 1 °C steps from 95 °C to 60 °C. Detection follows this cooling process, with the related SYBR green emissions reflecting the amplicon renaturation kinetics. Analysis of the resulting melting profiles allows determination of insertions and, hence, of the corresponding genotype of the genomic template.

The most consistent feature of Gilbert syndrome is a deficiency in bilirubin glucuronidation. The UGT1A gene is responsible for bilirubin catalytic enzyme production. The lower expression of the UGT1A gene is related to a TA insertion in the TATA box of the gene promoter (5).

The resulting PCR product differs from the wild-type gene by having a two-base insertion. This Tm analysis of PCR products allowed discrimination between the wild-type, homozygous, and heterozygous samples in an Italian family.

The PCR was performed as follows. To a final volume of 50 μL were added 1 × SYBR Green I Buffer (Molecular Probes); 5.5 mmol/L MgCl2; 200 μmol/L each dATP, dGTP, and dCTP; 400 μmol/L dUTP; 5 U of AmpliTaq Gold®; 1 U of AmpErase uracil-DNA-glycosylase; 50 ng of genomic DNA; 50 nmol/L of the forward primer (5′-ATTAACTTGGTGTATCGATTGGTTT-3′); and 300 nmol/L of the reverse primer (5′-CAGCAGGCCCAAGGACAA-3′). All reagents were purchased from PE Biosystems. The cycling conditions were as follows: 40 cycles of 50 °C for 2 min, 95 °C for 10 min, 94 °C for 15 s, and 60 °C for 1 min.

At the end of PCR, the dissociation protocol included a slow cooling from 95 °C to 60 °C in 20 min. During this time, the fluorescence was measured every 3 s, for a total of 400 data points. The reaction was performed on the 5700 Sequence Detection System equipped with the 5700 Sequence Detection System software. This application makes it possible to follow the melting profile for each amplicon; the software calculates the first derivative of the profile, making it possible to highlight the point at which the reassociation occurs (see Fig. 1).

More than 15 samples were run in quadruplicate and in different PCR runs. Unaffected DNA coming from unrelated subjects was used for the wild-type control samples.

To confirm the results, PCR products were sequenced or separated on 10% acrylamide gels.

The key to optimizing this assay is to minimize nonspecific amplification and primer-dimer formation. An effective hot start is provided here by the use of dUTP and uracil-N-glycosylase in the PCR. Although intended as a means of controlling carryover contamination from previously amplified DNA, the use of uracil-N-glycosylase and dUTP means that nonspecific primer extensions containing dU are cleaved up until the enzyme is heat-inactivated (6). This together with a “chemical” hot start given by the AmpliTaq Gold reduces the risk of primer-dimer formation.

For typing the UGT1A TATA box, 50 ng of genomic DNA was sufficient, and a primer optimization matrix was run in quadruplicate, using final concentrations of 50 and 300 mmol/L for each primer. No-template controls were run in the same assay to verify nonspecific PCR product formation. All of the tested 50/300, 50/50, and 300/50 (mmol/L) forward/reverse primer combinations did not give any nonspecific products. No fluorescence was detected up to the end of PCR (threshold cycle >40), whereas in the presence of target DNA, the PCR product was detectable after 27, 34, and 30 cycles, respectively. Lower cycle thresholds are related to a higher sensitivity of the test; the 50/300 (mmol/L) primer combination was chosen for this reason.

The 5700 Sequence Detection System allows “real-time PCR” by analyzing product formation cycle by cycle, thus allowing us to confirm the correct amount of starting genomic DNA. At the end of the reaction, the melting profile analysis was performed on the instrument. The melting profile of each sample was followed every 3 s; the first derivative at the flex point gave the right melting peak for each sample (9). To evaluate this experiment, each sample was run four times in the same PCR, and in three different PCR runs. The resulting Tm profiles are shown in Fig. 1.

This study presents an analysis of Gilbert syndrome in an Italian family: ~15 subjects from the same family were studied. The patient was a daughter born of consanguinous parents; together with the daughter and her parents, samples from all relatives were submitted to analysis. The coefficient of variation was calculated to validate the Tm test analysis; it ranged from 0.2% to 0.06%, thus allowing us to state that the tests were reproducible and reliable. Together with each sample studied, an unrelated wild-type control DNA was used in the same conditions.

The wild-type control and the homozygous patients (daughter and father) presented a clearly distinguishable difference in Tm (85.4 and 86.7°C, respectively). Several samples analyzed were heterozygous for a TA insertion; it was also possible to discriminate such samples because they showed Tm peaks with values between those of the wild-type and the mutant samples.

All PCR products were run on a 10% acrylamide gel to confirm the presence of insertions in both homozygous and heterozygous samples. The PCR reactions were also sequenced on an AmpliTaq Gold platform to confirm the Tm analysis pattern.

This newly developed technique allows screening of genomic variations such as single-nucleotide polymorphisms (9) and insertions.

Gilbert syndrome affects 6–12% of the population. This disease is often related to a TA insertion in the TATA box promoter of the UGT1A gene; affected heterozygous individuals present little or no visible manifestation, whereas homozygous subjects show mild jaundice and insufficient bilirubin clearance (5). To date, these kinds of analysis have been performed with different techniques such as sequencing, single-strand conformation polymorphism, and polyacrylamide gel electrophoresis of PCR products.

This technology is a suitable alternative to the standard PCR–single-strand conformation polymorphism method because it is straightforward and easier to perform, requires less DNA, is less time-consuming, and gives a good degree of automation compared with the need of pouring a gel for electrophoresis. We have developed an easy and inexpensive method of determining genotypes of double-base insertions that is not dependent on fluorescent probes and without post-PCR processing. This kind of

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**Fig. 1. Melting profiles.**

wt, wild-type control (green) with its relative Tm value on the x axis; het, heterozygous profile (blue); mut, mutant profile (father and daughter; red).
analysis is a very reliable test because it is reproducible and able to distinguish between wild-type and mutant samples, whether homozygous or heterozygous. The reproducibility of the method allowed us to test several subjects. All of the samples were successfully and unambiguously genotyped; the $T_m$ genotyping study was confirmed either by sequence or by polyacrylamide gel electrophoresis.

For further applications (i.e., single point mutations) this kind of genotyping assay would be an interesting method. The melting curve analysis is related to DNA sequence, GC content, and PCR product length (short amplicons are strongly preferred for reliable calling); taking such points into account, it should be easy to set up and perform other screening applications where, of course, a sequencing approach could always confirm the $T_m$ profile.

References

Rapid β-Globin Genotyping by Multiplexing Melting Temperature and Color, Mark G. Herrmann, Steven E. Dobrowolski, and Carl T. Wittwer\* (1) Department of Pathology, University of Utah, 50 N. Medical Dr., Salt Lake City, UT 84132; (2) Neo Gen Screening, Pittsburgh, PA 15220; * author for correspondence: fax 801-581-4517, e-mail Carl_Wittwer@hlthsci.med.utah.edu

Single-nucleotide polymorphisms have been identified by many different experimental approaches. The human β-globin gene has been genotyped by several methods, including PCR followed by restriction digestion (1,2), denaturing gradient gel electrophoresis (3), allele-specific amplification during PCR, and the ligase chain reaction (4). These methods require several hours and sometimes days for diagnosis. Recently, rapid-cycle PCR has been combined with real-time fluorescence monitoring to detect mutations by fluorescent probe melting point analysis for homogeneous genotyping in <1 h. Fluorescent melting point analysis is a technique that detects mutations by differences in the melting temperature ($T_m$) of fluorescent oligonucleotides hybridized to different alleles (5–7). Probes of a single color are usually used for genotyping. Four alleles at two different loci have been genotyped by multiplexing probe $T_m$s of a single color (8). However, there is a limit to how many alleles can be distinguished by differences in $T_m$. The ability to use multiple colored probes along with $T_m$ would greatly extend the power of monitoring PCR with fluorescence by allowing greater numbers of loci to be screened for mutations in one reaction. Exon 1 of the β-globin gene has >50 mutations, which produce various hemoglobinopathies (9). Hemoglobins S, C, and E are common and are routinely screened. Hemoglobin C (Hb C) results from a G-to-A mutation in the first nucleotide of codon 6, whereas hemoglobin S (Hb S) arises from an A-to-T mutation in the second nucleotide of this codon. Hemoglobin E (Hb E) results from a G-to-A mutation in the first nucleotide of codon 26. The close proximity of these three mutations allowed us to design a probe system that discriminated all genotypes using $T_m$ and two probe colors.

The human β-globin gene sequence (GenBank accession no. U01317) was used to design primers and probes for the amplification of a 214-bp segment containing exon 1 (Fig. 1a). Because of the high homology between the β-globin and δ-globin sequences, the primers (sense, 5’-GCAGGGCAGAGCCATCTA-3’; antisense, 5’-GGTCATTTGCTCTCCCTAAAGGTG-3’) were designed with 3’ and additional mismatches to δ-globin. In addition, because of the close proximity of the hemoglobin mutations, a unique combination of probes was designed to detect Hb S, C, and E alleles. Two probes were labeled with acceptor fluorophores, LightCycler Red 640 (LC Red 640) and LightCycler Red 705 (LC Red 705; Roche Molecular Biochemicals), as mutation detection probes. The third probe was a dual-labeled fluorescein donor probe that spanned the distance between the mutation detection probes. When annealed, resonance energy was transferred from each fluorescein label to either the LC Red 640- or the LC Red 705-labeled probes. The codon 6 detection probe (5’-CTCCTGTGAGAAGTCTTCGC-LC Red 640) completely matched the Hb S allele antisense strand. The codon 26 probe (LC Red 705-GTTGTTGTAAGGCGCTTGT-phosphate) completely matched the Hb E allele antisense strand. Both the LC Red 640 and LC Red 705 probes were obtained from Idaho Technology Biochem. The fluorescein-labeled probe was labeled with two fluorescein (F) molecules attached to the 5’ and 3’ ends (F-GTACTGGCCCTGTGGGCAGACGTTG-GATGAG-Operon). Fifty-five blinded samples of human genomic DNA were randomly selected from samples submitted to Neo Gen Screening for sickle cell hemoglobinopathy screening. The DNA (80–130 ng) was prepared from blots on filter paper (10) and had been genotyped previously by allele-specific cleavage and gel electrophoresis (2).