

index, Ret-Y (RET-H_e), appears to be clinically equivalent to the CHR and offers an attractive potential tool for the diagnosis and monitoring of iron-restricted erythropoiesis.

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Denaturing HPLC-Based Assay for Molecular Screening of Nondeletional Mutations Causing α -Thalassemias, Valentina Guida,^{1,2*} Alessia Colosimo,^{1,3} Mirella Fiorito,^{1,2} Enrica Foglietta,⁴ Ida Bianco,⁴ Giovanni Ivaldi,⁵ Marco Fichera,⁶ and Bruno Dallapiccola^{1,2} (¹ Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS)-Casa Sollievo Sofferenza (CSS), San Giovanni Rotondo and CSS-Mendel Institute, Rome, Italy; ² Department of Experimental Medicine and Pathology, University of Rome “La Sapienza”, Rome, Italy; ³ Department of Biomedical Sciences, University “G. D’Annunzio”, Chieti, Italy; ⁴ Centro Microcitemia Associazione Nazionale Microcitemia Italiana-ONLUS, Rome, Italy; ⁵ Human Genetic Laboratory, Galliera Hospital, Genoa, Italy; ⁶ Laboratorio di Patologia Genetica IRCCS Oasi Maria Santissima, Troina, Italy; * address correspondence to this author at: CSS-Mendel Institute, Viale Regina Margherita 261, 00198 Rome, Italy; fax 39-06-44160548, e-mail v.guida@css-mendel.it)

α -Thalassemias (OMIM 141850 and 141800; GenBank accession no. NT037887) are recessively inherited hemoglobin disorders caused by loss of function of either one of the two duplicated α -globin genes ($\alpha 1$ and $\alpha 2$), both located on chromosome 16p13.3 (1, 2). More than 95% of α -thalassemia phenotypes result from meiotic unequal recombinational events between the highly homologous $\alpha 1$ - and $\alpha 2$ -globin loci, which lead mainly to large genomic deletions (3–100 kb), which remove one to four α -globin genes, and rarely to α -gene triplication or quadruplication. Although less frequent, at least 48 different nondeletional mutations (including point mutations and deletions/insertions of a few nucleotides), mostly located in the $\alpha 2$ -globin gene, have also been reported as causative mutations of α^+ -thalassemia (3, 4). At present, molecular identification of this type of nucleotide mutation is carried out by specific PCR amplification of the $\alpha 2$ or $\alpha 1$

gene, followed by methods that have only a limited rate of detection (60–80%) and are technically demanding, such as single-strand conformation polymorphism (SSCP) analysis and denaturing gradient gel electrophoresis (5, 6), or are costly, cumbersome, and time-consuming, such as direct sequencing and reverse dot-blot analysis (7, 8).

We have evaluated the performance of a relatively simple and semiautomated technique, denaturing HPLC (DHPLC), that separates heteroduplex and homoduplex molecules on a stationary phase under partially denaturing conditions (9, 10). We tested a Transgenomic™ Wave DHPLC-based protocol for the molecular identification of α -globin gene nondeletional mutations in 50 wild-type individuals and 50 heterozygous carriers of Italian origin whose genes had previously been molecularly defined by restriction endonuclease digestion of PCR fragments and/or reverse dot-blot analysis.

Blood samples were collected from heterozygous individuals and healthy controls at the Centro Studi Microcitemie (Rome), the Galliera Hospital Genova, and the Oasi ONLUS Troina after informed consent was given. Genomic DNA was isolated from leukocytes in peripheral blood by salting out procedures (11). Purified DNA was solubilized in Tris-EDTA buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8) and stored at -20°C . Four overlapping primers (DHPLC1 through -4), were designed to amplify the entire region of both α -globin genes, including the 5' and 3' noncoding regions, at the same temperature. Because the third exon and the 3'-untranslated region of both α -globin genes are considered mutational hot-spot regions, we chose two pairs of specific primers located in regions of sequence dissimilarity, thus allowing the selective amplification and analysis of $\alpha 1$ or $\alpha 2$ genes. Furthermore, because the α -globin gene cluster is located within a GC-rich DNA region, which makes clean amplification quite difficult, we explored the use of different *Taq* DNA polymerases, also changing the composition of the Mg^{2+} -containing buffer. After testing several commercially available DNA polymerases, including AmpliTaq™ (PE Applied Biosystems), Taq Gold™ (PE Applied Biosystems), and Optimase DNA polymerase (Transgenomic; data not shown), we used Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen™). After empirical optimization, we performed PCR amplifications in a Gene Amp PCR system 9700 (PE Applied Biosystems); the PCR mixture (total volume, 25 μL) contained 250 μM each of the deoxynucleotide triphosphates, 100 ng of template DNA, 0.5 μM each of the primers, and 1.25 U of Platinum Taq DNA Polymerase High Fidelity (Invitrogen) in 1 \times reaction buffer [60 mM Tris-SO₄ (pH 8.9), 18 mM (NH₄)₂SO₄, and 2.0 mM MgSO₄]. To preserve the DNasep column, we replaced the Invitrogen buffer with the 1 \times Transgenomic Optimase buffer (2 mM MgSO₄), obtaining the same results. The PCR conditions consisted of an initial denaturation step (94 $^\circ\text{C}$ for 3 min), followed by 30 cycles at 94 $^\circ\text{C}$ for 30 s, 62 $^\circ\text{C}$ for 30 s, and 68 $^\circ\text{C}$ for 45 s, with a final extension step of 7 min at 68 $^\circ\text{C}$.

Each amplicon was heated at 95 $^\circ\text{C}$ for 5 min and cooled

slowly over 30 min at room temperature to promote heteroduplex formation. Between 3 and 5 μL of crude sample was then loaded on a DNasep column (Transgenomic) and subjected to DHPLC analysis in a WAVE DHPLC instrument (Transgenomic) as described previously (12). The reversed-phase gradient was formed by mixing buffer A (0.1 mmol/L triethylammonium acetate, pH 7.0) and buffer B (0.1 mol/L triethylammonium acetate, pH 7.0, containing 250 mL/L acetonitrile). PCR products were then separated (flow rate, 1.5 mL/min) through a 5% linear acetonitrile gradient. Oven temperature for optimal heteroduplex separation under partial DNA denaturation was determined by varying the oven temperature by $\pm 2^\circ\text{C}$ around the melting temperature suggested by the Transgenomic software 4.1.40 (Table 1).

Each abnormal elution profile was sequenced in both directions by use of a 310 Genetic Analyzer (PE Applied Biosystems) according to PRISM Dye Terminator and Dye Primer Cycle Sequencing chemistries.

Using this protocol we screened 12 different nondeletional mutations, including 11 point mutations, 1 five-nucleotide deletion, and 1 point mutation associated with the $-\alpha^{3,7}$ rearranged $\alpha 2/\alpha 1$ gene (Table 1). In addition, we used 50 Italian wild-type individuals as negative controls. All 12 different mutations analyzed showed distinct and recognizable elution peaks. A comparison of elution peaks from wild-type individuals and mutant heterozygous individuals for each DHPLC fragment is shown in Fig. 1. The chromatographic profiles for all individuals with an identical nondeletional mutation (i.e., Hb Hasharon, Hb Icaria, Hb Quong-Sze, $\alpha^{\text{HphI}}\alpha$, $\alpha^{\text{NcoI}}\alpha$, and $\alpha\alpha^{\text{NcoI}}$) were identical (data not shown), demonstrating the reproducibility of the method. One the most frequent Mediterranean nondeletional mutations, a single-nucleotide substitution at the start codon that abolishes a *NcoI* restriction site and is detected in both α -globin genes, was analyzed by DHPLC. Although both variants ($\alpha^{\text{NcoI}}\alpha$ and $\alpha\alpha^{\text{NcoI}}$) were located within the same

DHPLC2 fragment, we obtained different elution profiles at the same melting temperature (64.8 $^\circ\text{C}$), which allowed us to distinguish which of the two genes was mutated. Moreover, the presence or absence of a common neutral polymorphism ($\alpha 2 + 861\text{G}>\text{A}$), in conjunction with the $\alpha^{\text{TSaudi}}\alpha$ mutation on the same allele, changed the number and/or the shape of elution peaks compared with fragments containing a single mutation or polymorphism (compare the peaks in panels a, b, and c in Fig. 1).

Two different melting temperatures (63.8 and 64.8 $^\circ\text{C}$) were sufficient to rapidly detect all of the most common Mediterranean mutations, including $\alpha^{\text{NcoI}}\alpha$, $\alpha\alpha^{\text{NcoI}}$, $\alpha^{\text{HphI}}\alpha$, Hb Hasharon, Hb J-Sardegna, and Hb Icaria. For this reason, we suggest that the initial screening of any new Italian α -thalassemia patient should be performed on fragments DHPLC1, DHPLC2, and DHPLC4 $\alpha 2$, which contain the vast majority of α -globin nondeletional mutations found in the Italian population. Overall, the DHPLC-based protocol produced neither false-negative nor false-positive results among 200 chromosomes. In contrast to traditional molecular techniques for the detection of unknown nondeletional α -globin mutations (such as denaturing gradient gel electrophoresis, SSCP analysis, and direct sequencing), our DHPLC-based protocol was not affected by the extensive sequence homology and high GC content of the α -globin loci (5, 6). Furthermore, compared with the above-cited methods, DHPLC is more rapid and has lower costs because, once preliminary work has been carried out to establish optimal PCR conditions, no special manipulations or implementations are required after amplification. Our data are concordant with a previous study of mutational screening carried out on the Notch 3 gene, which contains an extremely high GC content, that showed a higher sensitivity of DHPLC compared with SSCP and heteroduplex analysis (13).

Different from other equally simple strategies, such as reverse dot-blot hybridization, DHPLC-based screening is not limited to the identification of previously reported

Table 1. DHPLC analysis conditions and analyzed mutations.

Fragment	Length, bp	Primer pairs, 5'-3'	Oven temperature, $^\circ\text{C}$	Buffer B, %	Mutations analyzed	No. of samples analyzed
DHPLC1	328	gcgccccaagcataaacctcg aggctcttggtggtgggagg	64.8	60-70	$\alpha\alpha^{\text{NcoI}}$ $\alpha^{\text{NcoI}}\alpha$ $\alpha\alpha^{\text{HphI}}$	10 6 2
DHPLC2	275	accctcaaccgtcctggccc cgccgctcacctgaagttg	63.8	58-68	Hb Hasharon Hb J-Sardegna Hb Winnipeg Hb Toulon	6 1 1 1
DHPLC3 $\alpha 1$	245	gcgacctgcacgcgcaaa gtgaactcggcgggagggtg	65.0	58-68		
DHPLC3 $\alpha 2$	238	gcgacctgcacgcgcaaa gtgaactcggcgggagggtg	65.9	57-67		
DHPLC4 $\alpha 1$	361	ctgggcccctcgcccactga ccctatccagctgcagagaggtt	65.0	58-68	Hb J-Camaguay	1
DHPLC4 $\alpha 2$	374	gggcccgaactgaccctcttc catgcagctgcagagaggtcc	64.8	61-71	Hb Icaria $\alpha^{\text{TSaudi}}\alpha$ Hb Quong Sze Hb Costant Spring	4 13 3 2

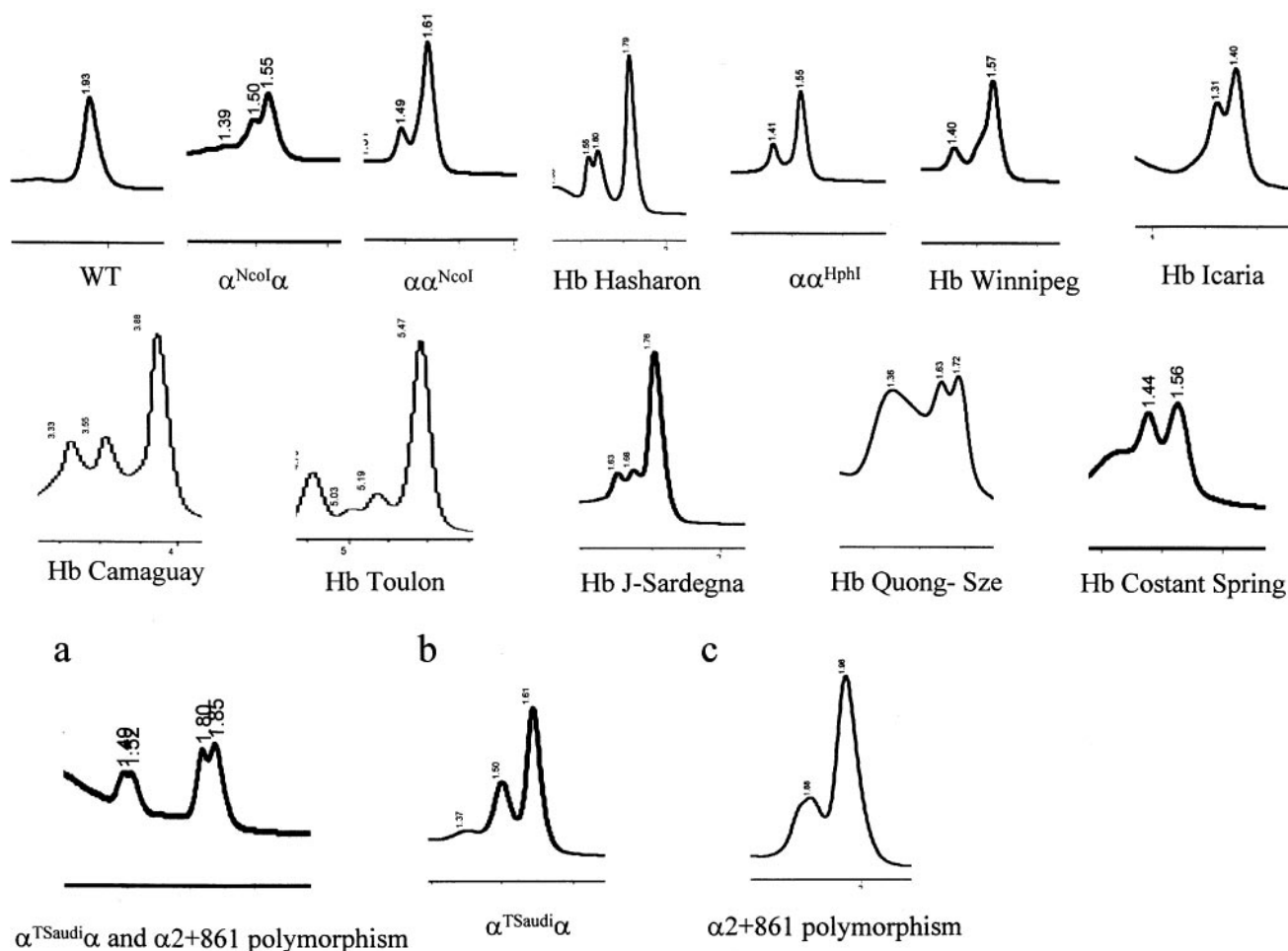


Fig. 1. DHPLC chromatograms of α -thalassemia mutations and polymorphisms.

For descriptions of the peaks in a, b, and c, see the text.

mutations but also allows the molecular characterization of unknown point mutations and small deletions/insertions.

In conclusion, we show that DHPLC is a reliable, sensitive, and specific screening method to rapidly detect the most common nucleotide mutations described in the Italian population. Many individuals with different or identical mutations can be simultaneously screened by this method. The analysis time of 2.5 min/sample offers the possibility of semiautomated analysis of 96 samples in <12 h. As such, this semiautomated method, coupled with the low costs of analysis per sample, would be suitable for rapid, large-scale mutational screening of frequent and rare mutations. In fact, although mutations affecting each globin gene are usually population specific, the widespread movement of people among communities favors a method that can identify a heterogeneous spectrum of both common and rarer α -globin variants. In addition, molecular analyses are essential for the accurate diagnosis and subsequent risk ascertainment of double-heterozygous carriers for α - and β -globin alleles. In fact, in individuals who are heterozygous for β -thalassemia,

concurrent α -thalassemia can mask the classic hematologic manifestations and make the β -thalassemia carrier state less apparent, leading to errors in genetic counseling (3, 14).

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Plastic versus Glass Tubes: Effects on Analytical Performance of Selected Serum and Plasma Hormone Assays, Carol M. Preissner, William M. Reilly, Richard C. Cyr, Dennis J. O’Kane, Ravinder J. Singh, and Stefan K.G. Grebe* (Endocrine Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN; * address correspondence to this author at: Endocrine Laboratory, Hilton 730C, Mayo Clinic, 200 1st St, SW, Rochester, MN 55905; fax 507-284-9758, e-mail grebs@mayo.edu)

During the last decade, plastic blood collection tubes have been progressively replacing glass tubes. Plastic tubes are not only less expensive but also safer than glass tubes, because they are less likely to break. Unfortunately, it is frequently difficult or impossible for individual laboratories to obtain comprehensive data on the equivalence of replacement plastic tubes vs their original glass counterparts. This is a particularly important issue for many endocrine assays, especially peptide hormones. These often degrade rapidly and can adsorb to a variety of surfaces (1–7). Changing from glass to plastic tubes can also be problematic for analytes that are regarded as stable. An example of the latter can be seen in therapeutic drug monitoring, where plastic tubes have been shown to influence the measured concentrations or stabilities of several drugs (8–10). Similar concerns may apply to low-molecular-weight hormones, such as steroid hormones and biogenic amines. These are increasingly assayed by HPLC, gas chromatography–mass spectrometry, or liquid chromatography–tandem mass spectrometry. It is conceivable that low-molecular-weight organic substances released by plastic tubes could interfere in some of these assays (11) and that small changes that are not

detected by immunoassays would change the results of more specific methods. Finally, in certain situations, such as serial monitoring of tumor markers, even minor discrepancies between glass and plastic tubes may gain significance during changeover from one type of collection to the other.

We designed the present study to give a reasonable representation of the range of analytes and analytical methodologies used in our laboratory, with particular emphasis on peptide hormones because of their known instability. We selected the following analytes:

- (a) Five peptide hormones—adrenocorticotropin (ACTH), insulin, insulin-like growth factor I (IGF-I), parathyroid hormone (PTH), and human growth hormone (hGH)—covering a range of known stabilities, physicochemical properties, and molecular weights;
- (b) Two glycoproteins, human β -chorionic gonadotropin (β -hCG) and cancer antigen-125 (CA-125), an ovarian cancer tumor marker; and
- (c) Two steroid hormones, cortisol and 17-hydroxyprogesterone (OHPG).

The OHPG assay is an in-house, extracted liquid chromatography–tandem mass spectrometry method with an analytical range of 0.33–330 nmol/L, interassay CV of 2.6–10%, and recoveries of added analyte and dilution linearities of 96–118%. The PTH assay is an in-house intact molecule manual direct chemiluminometric assay that uses a pair of in-house-produced polyclonal antibodies with <30% cross-reactivity with PTH_{7–34} and no cross-reactivity with more C-terminal fragments. Assay CVs across the analytical range were 5–18%. The other assays are automated direct immunoassays performed on the Beckman-Coulter Access platform (insulin, β -hCG, cortisol, and hGH), the Nichols Advantage system (ACTH and IGF-I), and the Ortho-Diagnostic Vitros ECi-analyzer (CA-125). All automated assays conformed to the respective manufacturers’ performance specifications, which we verified in house. The β -hCG assay uses a combination of a mouse monoclonal antibody to the β -subunit and a polyclonal alkaline phosphatase-linked rabbit anti-total hCG antibody for analyte detection, and goat anti-mouse-antibody-coated paramagnetic beads for separation. The limit of quantification is 0.5 IU/L.

We used a randomized schedule of tube order, generated by our statistics department, for all blood draws to control for any draw-order bias. For all analytes except ACTH, we drew blood from 25 volunteers (age range, 24–57 years; 10 males and 15 females) into serum separator (SS) glass tubes (Becton Dickinson) and into SS plastic tubes manufactured by Becton Dickinson (Vacutainer Tube; designated as type A), Monoject (Sherwood Corvac tube; designated as type B), and Greiner (Greiner Vacuette tube; designated as type C). Except for the insulin hemolysis and ACTH portions of the study (see below), all tubes had the same lot numbers. We froze an aliquot from each tube at -20°C and kept the remainder at 4°C for 1 week before also freezing it. All aliquots were thawed and