High-speed detection of the G894T polymorphism in the exon 7 of the eNOS gene by real-time fluorescence PCR with the Light-Cycler

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High-Speed Detection of the G894T Polymorphism in Exon 7 of the eNOS Gene by Real-Time Fluorescence PCR With the Light-Cycler

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In endothelial cells nitric oxide (NO) is synthesized by endothelial-nitric oxide synthase (e-NOS), constitutively expressed and encoded by a 26-exon gene, located on chromosome 7q35-36. The prevalence of the T rare variant of the G894T polymorphism in exon 7 of the e-NOS gene (Glu→Asp amino acid substitution) has been reported to be significantly higher in patients with coronary spasm and coronary artery disease. To date G894T polymorphism detection is performed by PCR-RFLP assay. In order to establish a high-speed genotyping method, we have taken advantage of the Light Cycler instrument, a thermal cycler that combines rapid-cycle DNA amplification with a real-time fluorescence monitoring. This technology is based on hybridization of the adjacent fluorescently labeled probes with PCR products. This methodology is considered more accurate and less time-consuming than conventional PCR-RFLP assay. To validate this technique we genotyped 270 healthy subjects. The results were consistent with those obtained from PCR-RFLP assay.

KEY WORDS: Light Cycler; real-time PCR; e-NOS gene; G894T polymorphism.

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INTRODUCTION

Nitric oxide (NO), a potent regulator of the cardiovascular system, is synthesized from L-arginine by a family of three NO synthases (NOS): neuronal (n-NOS, type I), inducible (i-NOS, type II), and endothelial (e-NOS, type III) (Wang and Marsden, 1995). In endothelial cells, NO is synthesized by the e-NOS, constitutively expressed, encoded by a 26-exon gene located on chromosome 7q35-36 (Marsden *et al.*, 1993). Endothelium-derived NO controls the vascular tone, inhibits platelet (Radomski *et al.*, 1987) and leucocyte adhesion to vascular endothelium (Kubes *et al.*, 1991), inhibits smooth muscle cell migration (Sarkar *et al.*, 1996) and growth (Garg and Hassid, 1989), and limits the oxidation of atherogenic low-density lipoproteins (Hogg *et al.*, 1993). These mechanisms suggest an important atheroprotective role, beyond its effect on vessel tone and blood pressure, and that an alteration in the vascular NO system activity could contribute to the pathogenesis of atherosclerosis. The e-NOS human gene exhibits many polymorphisms, some of which seem related to a significant proportion of the variance of NO plasmatic levels. A point mutation of guanine to thymine at nucleotide 894 in exon 7 of the eNOS gene, which results in the replacement of glutamic acid by aspartic acid at codon 298 (Glu298Asp) has been described (Hingorani *et al.*, 1995). It has been demonstrated that this polymorphism is not silent and may possibly be perceived as a target for cleavage (Tesauro *et al.*, 2000). Evidence has been presented that this polymorphism is associated with coronary spasm (Yoshimura *et al.*, 1998), acute myocardial infarction, and coronary artery disease in Caucasians (Hingorani *et al.*, 1999).

EXPERIMENTAL PROCEDURE

To date, G894T polymorphism detection for e-NOS is performed by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) analysis through digestion with the MboI restriction enzyme and resolution by electrophoresis on agarose gel (Hingorani *et al.*, 1999). In order to establish a high-speed and facile genotyping method, we have taken advantage of the Light Cycler (Roche Diagnostics), a microvolume fluorometer integrated with a thermal cycler that combines rapid-cycle DNA amplification with real-time fluorescence monitoring (Wittwer *et al.*, 1997). This technology is based on hybridization of amplicon-specific oligonucleotide probes, labeled with adjacent fluorophores capable of fluorescence resonance energy transfer (FRET) introduced in a thermal cycler (Light Cycler) that uses glass capillaries and hot air for heating. The probes are designed to hybridize to the same strand between the two unlabeled primers. A detection probe (Sensor probe), which matches the rare allele sequence, is labeled at its 3′-end with fluorescein (FLU). Moreover, a second hybridization probe (Anchor probe) is labeled at the 5′-end with a Light Cycler red fluorophore (LC

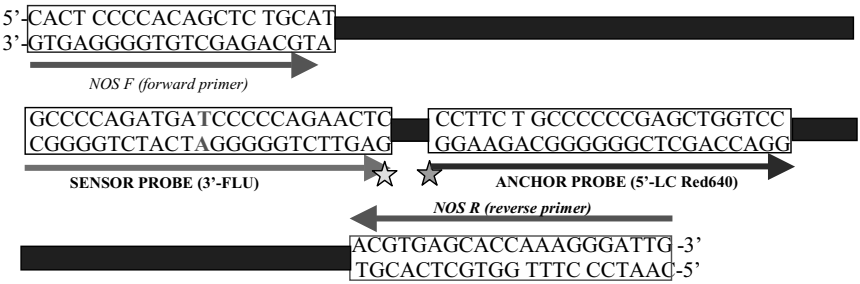
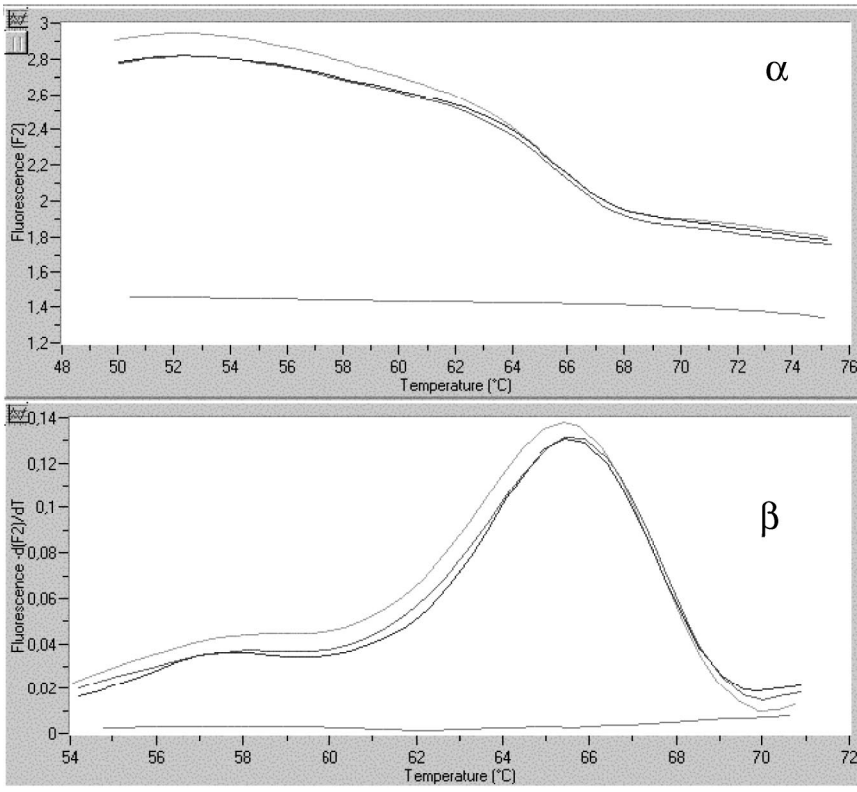


Fig. 1. Schematic showing positions of primers and hybridization probes for amplification and genotyping of eNOS G894T polymorphism site. An amplicon that spans the area of the polymorphism site is created with the unlabeled primers. Two hybridization probes anneal to the same strand internal to the unlabeled primers. The Sensor probe is labeled at its 3′-end with fluorescein (FLU), and the Anchor probe is labeled at its 5′-end with a Light Cycler red fluorophore (LC Red 640).

Red 640) (Fig. 1). When both probes hybridize in close proximity (in a head-to-tail manner), fluorescence energy transfer between the donor fluorophore (FLU), which is excited by a LED (light emitting dye) light source of the instrument ($\lambda = 530$ nm), and the acceptor fluorophore (LC Red 640) occurs. The acceptor fluorophore emits light of a longer wavelength ($\lambda = 640$ gm), which is detected in specific channels of the instrument.

For e-NOS G894T polymorphism genotyping, genomic DNA was isolated and purified from EDTA blood using QIAamp spin columns according to the manufacturer's instructions (QIAamp Blood Kit, Qiagen, GmbH, Hilden, Germany). Light Cycler (LC) amplification is performed in a final volume of 20 μL in the LC-glass capillaries. The reaction mixture consisted of 12.4 μL of H_2O, 0.8 μL of $MgCl_2$ (25 mM), 10 pmol of each primer, NOSF (5′-CACTCCCCACAGCTCT GCAT-3′) and NOSR (5′-CAATCCCTTTGGTGCTCACG-3′), 4 pmol of Anchor probe, 4 pmol of Sensor probe, and 2 μL of DNA-Master hybridization probes (Reaction Buffer). Two microliters of genomic DNA (50–200 ng/μL) were used for amplification. Fluorescently labeled hybridization probes were synthesized by TIB Molbiol (Berlin, Germany). The Anchor probe, 5′-LC Red 640-CCTTCTGCCCCCCGAGCTGGTCC-3′-P, was 5′ labeled with the LC-Red 640 fluorophore and phosphorylated (P) at its 3′-end to prevent probe elongation by the Taq polymerase. The detection probe, 5′-CCCCAGATGATCCCCCAGAACTC-3′, was labeled with fluorescein (FLU) at its 3′-end.

Cycling conditions for e-NOS G894T polymorphism detection include an initial denaturation at 95°C for 30 s, followed by 45 cycles with a fast denaturation at 95°C with an incubation time of 0 s, annealing at 62°C for 10 s and extension at 72°C for 12 s with a ramping time of 20°C/s. After amplification, melting curves were generated following a fast denaturation of the reaction at 95°C with an

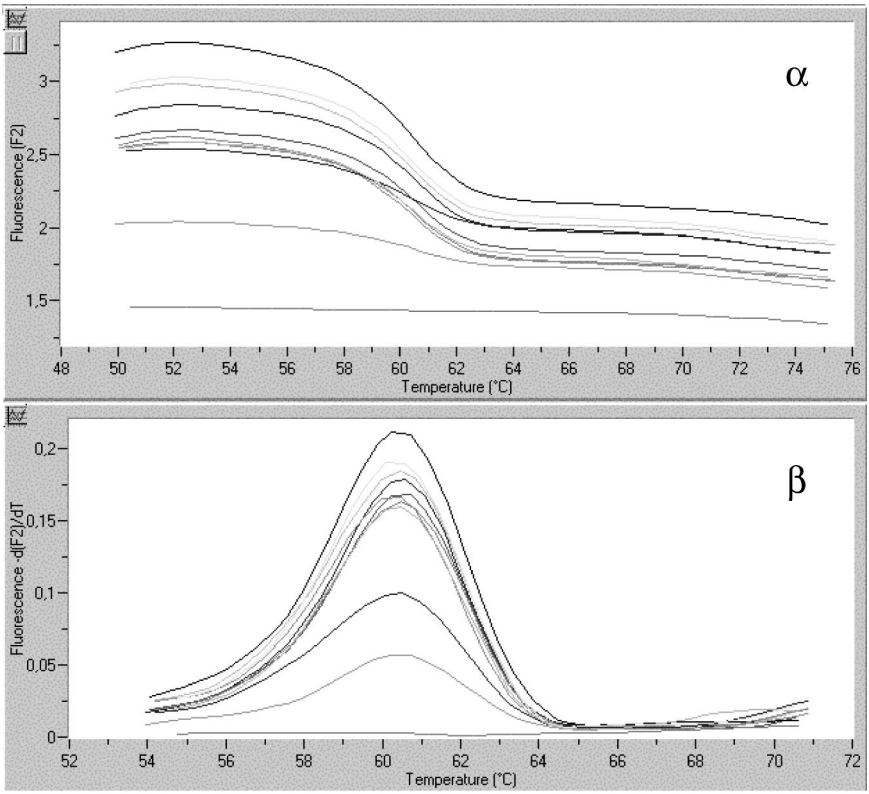


(A)

Fig. 2. Melting curves (α) are converted to melting peaks (β) by plotting the negative derivative of fluorescence with respect to temperature ($-dF/dT$). (A) The typical melting curve pattern, obtained when the Sensor probe hybridizes with the specific DNA sequence (894T rare allele), is a single melting peak at a temperature of 65.5°C. The picture shows a homozygous genotype for T rare allele (TT). (B) For the common allele (894G) there is a mismatch between the PCR product and the Sensor probe, which leads to strand instability and consequent earlier melting. The result is a single melting peak at a temperature of 60.5°C. The picture shows a homozygous genotype for the common G allele (GG). (C) Heterozygous patients show two melting peaks, one at 60.5°C and one at 65.5°C. The picture shows a heterozygous genotype (GT).

incubation time of 0 s, holding the sample at 52°C for 30 s and then slowly heating the sample to 75°C with a ramp rate of 0.1°C/s. Melting curves were converted to melting peaks by calculating the negative derivative of the fluorescence with respect to temperature ($-dF/dT$) against temperature. This plot allows distinction of the common allele from the rare one.

The typical melting curve pattern, obtained when the Sensor probe hybridizes with the specific DNA sequence (894T rare allele), is a single melting peak at

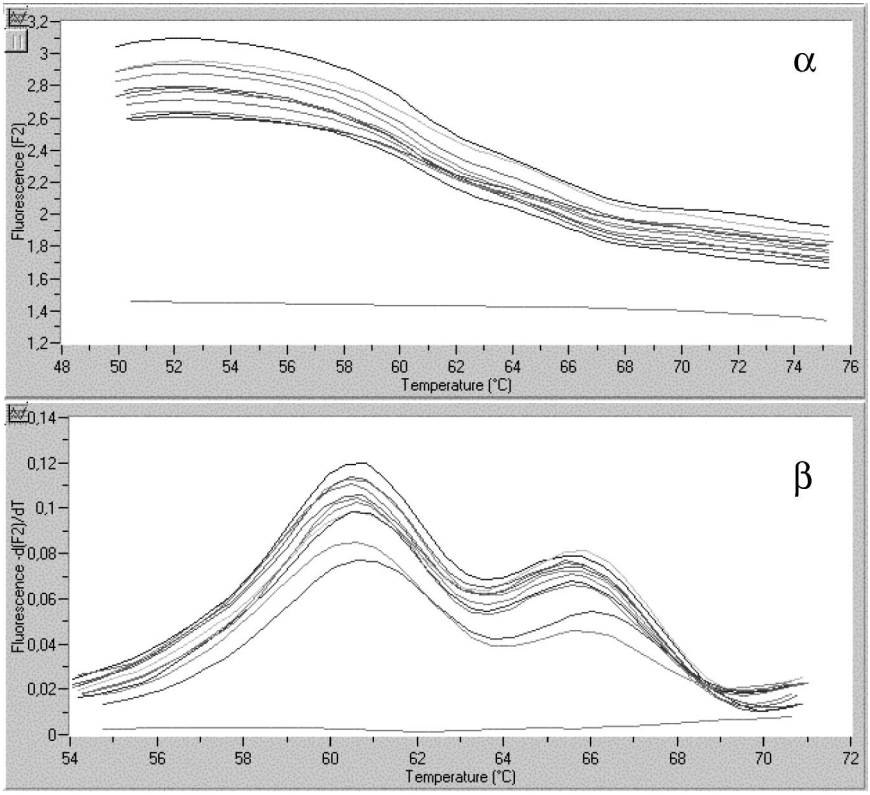


(B)

Fig. 2. (*Continued*).

a temperature of 65.5°C (Fig. 2(A)). For the common allele (894G) there is a mismatch between the PCR product and the Sensor probe, which leads to strand instability and consequent earlier melting. The result is a single melting peak at a temperature of 60.5°C (Fig. 2(B)). Heterozygous patients show two melting peaks (60.5 and 65.5°C) (Fig. 2(C)).

To validate this technique, we genotyped 270 healthy subjects recruited from blood donors: 61 subjects were homozygous for the mutant 894T allele (TT, 22%), 172 were heterozygous (GT, 64%), and 145 were homozygous for the G allele (GG, 54%). The 894T allele frequency was 0.33 (Table I). The genotypes were easily and clearly identified by generation of characteristic fluorescence melting peaks. We have compared this technique with the results obtained with a conventional method (PCR-RFLP): in each case, identical genotypes were obtained.



(C)

Fig. 2. (*Continued*).

This methodology gives more accurate results than conventional PCR-RFLP-based assay because it minimizes PCR contamination concerns related to sample handling and does not require digestion of PCR products with restriction enzymes and/or fragment separation on gels; it also does not require the use of hazardous reagents such as ethidium bromide and permits high-speed genotyping (i.e., 30

Table I. Distribution of Genetic Polymorphisms of the eNOS G894T Polymorphism

eNOS 894	TT	GT	GG	T	G
Genotype	61 (22%)	172 (64%)	145 (54%)		
Allele frequency				0.33	0.67

(Healthy Subjects $n = 270$).

samples can be analyzed in less than 2 h, instead of 8 h needed for PCR-RFLP assay).

In conclusion, this assay has shown its reliability and rapid practicability. It is especially suitable for molecular diagnostic routine laboratories, where a large number of samples must be processed.

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