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Note

Rapid Genotyping of Human ERCC1 Exon 4 Polymorphism With Fluorescence Analysis Using Fluorophore-Labeled Hybridization Probes and a LightCycler

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INTRODUCTION

Because of their abundance, and because many effectively dichotomize human populations, single nucleotide polymorphisms (SNPs) are attractive tools for studying gene-phenotype interactions, for instance in commonly occurring diseases. Although our understanding of linkage disequilibrium is still incomplete (Abecasis *et al.*, 2001), it is generally assumed that a SNP can serve as a marker for the surrounding DNA region in outbred populations. However, the size of the regions may well vary considerably between SNPs and populations and discontinuities may occur.

To explore and utilize SNPs in such studies it is necessary to develop quick, reliable, high-throughput typing methods. One approach is the development of solid-state arrays (Fan *et al.*, 2000), which can be used to type thousands of SNPs in a person's DNA. The approach allows for the coverage of the complete genome with an average distance between SNPs of a few 100 kb. However, the statistical problems are large, and the issue of mass significance will be difficult to deal with.

We have taken a different approach. On the assumption that the same genes influence rarely occurring familial diseases and the commonly occurring "sporadic" diseases we have chosen areas around already defined genes and studied those

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with LightCycler technology in relation to disease occurrence in outbred populations (Dybdahl *et al.*, 1999; Vogel *et al.*, 2001). The technique typically allows for the typing of one SNP in each assay, but has a fast turn-around time. We have chosen a SNP, designed primers and probes for it, had them synthesized, optimized PCR and temperature profiling for the reaction, typed around 200 persons for the SNP, and analyzed the results in a matter of 3 weeks. The time can be further reduced, if fast access to synthesis of primers and probes is available. Thus, with this technique the choice of markers can be a dynamic process.

In this paper we illustrate the use of LightCycler technology by presenting a single-step assay for a polymorphism in human ERCC1 exon 4.

MATERIALS AND METHODS

Primers and Probes

The forward primer was 5′-GGCCCTGTGGTTATCAAGG-3′ and the reverse primer was 5′-TTCCTGAAGTCTGGGGTGG-3′. Hobolth DNA, Hillerod, Denmark, synthesized the primers. The mutation site was covered by a fluorescein-labeled sensor probe complementary to the wild-type allele: 5′-CGCAACGTGC CCTGGGAAT-3′-Flu. The adjacent anchor probe was labeled with Lightcycler-Red-640: 5′-LCRed640-TGGCGACGTAATTCCCGACTATGTGCTG-p. The 3′end of the anchor probe was phosphorylated to prevent probe elongation. The probes were synthesized by TIB-MolBiol, Berlin, Germany.

PCR reactions were performed in a final volume of 20 μL in LightCycler capillaries. The reaction mixture contained 0.5 uM of each primer, 45 nM of anchor and sensor probes, 3.5 mM $MgCl_2$, approximately 7 ng genomic DNA, and 2 μL LightCycler DNA master hybridization probe buffer (Roche Molecular Biochemicals, Manheim, Germany). This buffer contained Taq DNA polymerase and dNTP, and also contributed to the $MgCl_2$ concentration.

The temperature cycling consisted of denaturation at 95°C for 2 s, followed by 46 cycles consisting of 2 s at 95°C, 10 s at 57°C, and 30 s at 72°C. The last annealing period at 72°C was extended to 120 s. After denaturation at 95°C for 2 s the melting profile was determined by a temperature ramp from 50°C to 95°C with a rate of 0.1°C/s. Each run contained 4 control samples and 28 unknown samples. The controls were: water, homozygous wild-type DNA, heterozygous DNA, and homozygous rare-allele DNA. Genotypings of the DNA controls and several other DNA samples were also performed with conventional PCR technique, followed by determination of a restriction fragment length polymorphism caused by the SNP. The two techniques gave identical results (results not shown).

RESULTS AND DISCUSSION

The LightCycler (Roche Molecular Biochemicals) is a microvolume fluorometer integrated with a thermal cycler and combines PCR with real-time fluorescence monitoring (Ahsen *et al.*, 1999; Bollhalder *et al.*, 1999; Nauck *et al.*, 1999; Wittwer *et al.*, 1997). In a LightCycler, the appearance of a specific PCR product can be monitored by hybridization probes, which are designed to bind to the amplified DNA next to each other. The 3′ end of one probe is labeled with fluorescein, whereas the adjacent 5′ end of the other probe is labeled with LightCycler Red. When both probes hybridize to the same PCR product, fluorescence resonance energy transfer occurs, that is excitation of the fluorescein leads to emission of light from LightCycler Red. Thus, the accumulation of amplified DNA can be followed by measuring the intensity of the light emitted from LightCycler Red relative to the light obtained from fluorescein. Furthermore, the two probes are deliberately designed such that the sensor probe is released first from the PCR product, when temperature is increased. Consequently, as the sensor probe spans the polymorphic site, its melting temperature will reveal which allele(s) is present in the amplified DNA. In general, a single base mismatch will lead to about 5°C reduction in melting point.

Figure 1 shows an example of the accumulation of amplified DNA as a function of the number of cycles. Figure 2 shows the slope of the melting curves as a function of temperature. Homozygous wild-type DNA, which formed a perfect match with the sensor probe, produced a single high-melting peak; DNA homozygous for the variant allele produced a single peak melting at lower temperature; and, heterozygous DNA produced two peaks.

Most LightCycler results are completely unambiguous, as long as adequate amounts of genomic DNA are included in the test. However, if the amount of DNA is too small, the temperature curve becomes too flat, and the result is difficult to call. Moreover, to ensure adequate detection of heterozygotes, it is necessary that a sufficient number of genomes are present and participate in the PCR. Thus, excessive economy with the DNA should be avoided. We generally prefer to work with at least 1000 genome equivalents in a tube.

In our experience LightCycler assays for most SNPs are easy to set up. However, in one or two cases results have been negative after several rounds of optimization. In these instances we have found it easier to switch to another nearby SNP, rather than starting an extensive search for an explanation.

Finally, in some assays the melting curve of the high-melting allele tends to produce a background smear at lower temperatures. This background is rarely strong enough to be taken for a low-melting allele, but in our experience it can be removed or at least reduced significantly by manipulating the cooling of the PCR reaction immediately before measurement of the melting curve. Thus, a slower

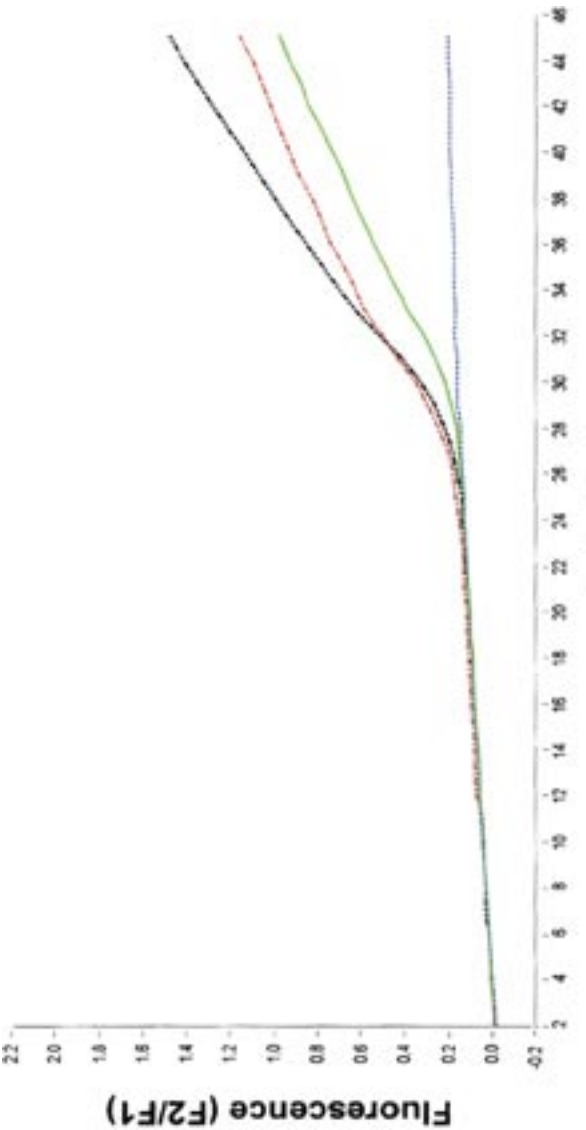

Fig. 1. Increase in relative fluorescence as the DNA products accumulate with each PCR cycle (···) Water; (– –) homozygote for the rare allele (ERCC1 exon4^{GG}); (–·–) homozygote for the wild-type allele (ERCC1 exon 4^{AA}); (—) heterozygote (ERCC1 exon 4^{AG}).

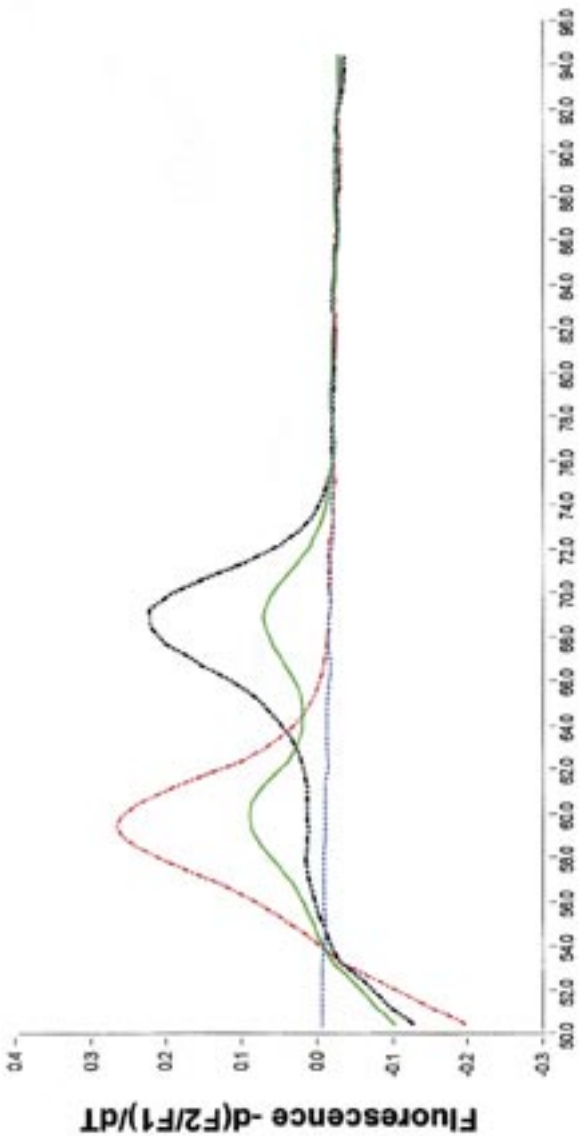

Fig. 2. Slopes (minus first derivatives) of the melting curves. Designations as in Fig. 1.

cooling from approximately 65°C down to 50°C after the final denaturation often helps.

Assays with LightCycler provide reliable high-speed throughput. One person can routinely make and run an assay with 28 unknown samples in about 2 h. The maximal capacity of the apparatus is about 1 run/h. Finally, the adaptation of the method to new SNPs is fast. We generally calculate that the design, ordering, synthesis, and shipment of primers and probes take about a week, and optimization of the PCR reaction and melting curve takes another week. Thus, the method is ideally suited for consecutive typing of a number of SNPs in pursuit of a gene in moderate-sized population samples.

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