Multiplex Molecular Diagnosis of MEFV Mutations in Patients with Familial Mediterranean Fever by LightCycler Real-Time PCR, Elena Rossou1,2,3, Anastasia Kouvatsi3, Charalampos Aslanidis4, and Constantinos Deltas1,2,* (1 Department of Biological Sciences, University of Cyprus, Nicosia, Cyprus; 2 Department of Molecular Genetics C', The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus; 3 School of Biology, Aristotle University of Thessaloniki, Thessaloniki Greece; 4 Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany; * address correspondence to this author at: Department of Biological Sciences, University of Cyprus, Kallipoleos 75, 1678 Nicosia, Cyprus; fax 357-22-892881, e-mail deltas@ucy.ac.cy)

Familial Mediterranean fever (FMF) is an autoinflammatory disease inherited as an autosomal recessive condition and characterized by recurrent episodes of fever, abdominal disease inherited as an autosomal recessive condition and characterized by recurrent episodes of fever, abdominal pain, pleuritis, and arthritis and significantly higher risk for amyloidosis amyloid A protein I with significantly higher risk for amyloidosis amyloid A protein I with significantly higher risk for renal failure. Many researchers have associated the M694V mutation as well as the α allele of serum amyloid A protein I with significantly higher risk for amyloidosis (6).

FMF is very prevalent in non-Ashkenazi Jews, Armenians, Arabs, and Turks, all of whom have ancestors of Mediterranean origin. The carrier frequency in these populations can reach up to 1:5; rendering it the most frequent autosomal recessive condition. Currently, it is considered frequent in other Mediterranean countries, including Cyprus (7–11). The gene associated with FMF, MEFV, which encodes pyrin/marenostrin, a protein of 781 amino acids (12, 13), belongs to the pyrin family of genes that play a role in autoinflammatory diseases and inflammatory pathways (14, 15). More than 40 mutations have been identified, but 4–6 mutations usually account for a high percentage of MEFV chromosomes in different ethnic populations, emphasizing the importance of molecular epidemiologic studies for identifying the various molecular defects in the population of interest. The detection of known mutations in MEFV can be accomplished by various methods, including direct DNA sequencing. These methods are time-consuming and prone to carryover PCR contamination because of the repetitive work in a diagnostic setting. Using fluorescence resonance energy transfer (FRET) on a LightCycler® instrument (Roche), we developed a rapid method that detects point mutations and small deletions by use of fluorescent hybridization probes. Because of the close proximity of several mutations on exon 10, we designed a single pair of fluorescent probes with multiplex melting profiles that permits the simultaneous examination of 5 mutations: K695R, M694V, M694I, M694del, and I692del.

We used DNA from patients diagnosed with FMF in our laboratory and samples from carrier relatives and the general population to serve as controls. Oligonucleotide primers and hybridization probes were synthesized (Tib Molbiol) for melting curve analysis (Table 1).

Fluorescence monitoring during real-time amplification with hybridization probes is based on the concept that a fluorescence signal is generated if FRET occurs between 2 adjacent fluorophores that are usually designed to bind on 1 amplicon strand (16–19). Amplification was performed in 20-μL volumes in glass capillaries, in the presence of 1.6 μL of 25 mM MgCl2 and 1 μL each of anchor and mutation probe (4 μM; details of the PCR protocols are shown in Table 1 of the Data Supplement.

### Table 1. PCR primer and LightCycler probe sequences used in this work for mutation detection.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide change</th>
<th>Exon</th>
<th>Primer name</th>
<th>Sequence, 5'–3'</th>
<th>Annealing T, °C</th>
<th>MgCl2, mM</th>
<th>PCR size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>F479L</td>
<td>1437C→G</td>
<td>5</td>
<td>479 forward</td>
<td>GCTGAAGCAGCGGCTGCAGAGG</td>
<td>55</td>
<td>3.25</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>479 reverse</td>
<td>GGGCGATGTCCTGGAATACGCCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>479 anchor</td>
<td>GAGCATTTCCTTGAGGCT-Flu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>479 detector</td>
<td>Red640-CTGGAGGACGTGGGCCAGATGGTGCGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V726A</td>
<td>2177T→C</td>
<td>10</td>
<td>726 forward</td>
<td>ATAGACAGAAAGGGAACATG</td>
<td>56</td>
<td>3.25</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>726 reverse</td>
<td>GGGCGAGGCCCACAGAGAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>726 anchor</td>
<td>TGGACATCAAGGCTGGAAGCA-Flu</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>726 detector</td>
<td>Red640-CTCCTTTTACATGTGACAGCCAGATC</td>
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<td></td>
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<tr>
<td>I692del</td>
<td>2074–2076del</td>
<td>10</td>
<td>694 forward</td>
<td>TTGGAGACAAGCAGCAT</td>
<td>47</td>
<td>3.25</td>
<td>201</td>
</tr>
<tr>
<td>M694V</td>
<td>2080A→G</td>
<td>10</td>
<td>694 reverse</td>
<td>CTCTGATGTCACAGAAG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M694I</td>
<td>2082G→A</td>
<td>10</td>
<td>694 anchor</td>
<td>GTTGGTGAGAATAAGATGAGAA-Flu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M694del</td>
<td>2078–2080del</td>
<td>10</td>
<td>694 detector</td>
<td>Red640-TGAGTCACAGGCGTCCAGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* In the sequences of the anchor probes, underlining indicates the substituted nucleotides and bold italics indicate the 2 deletions of 3 nucleotides each. For nucleotide numbering, +1 is the adenosine in the first ATG codon.

b FLU, fluorescein.

ATA encodes amino acid 692, and ATA encodes amino acid 694.
that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue9/ and are also available from the authors.

The probes hybridize head-to-tail, bringing the 2 fluorescent dyes into proximity. Fluorescence was monitored while the temperature was slowly increased; the fluorescence decreased when one of the probes melted off and the fluorescent dyes were no longer in proximity. The typical melting pattern with the wild-type DNA sequence is a single melting peak at a characteristic, reproducible temperature. In cases with homozygous mutations, the anticipated mismatch leads to hybrid instability and consequent earlier melting. The result is a single melting peak at a characteristic, lower temperature, whereas samples from patients with heterozygous mutations show 2 melting peaks.

All results generated with LightCycler technology were tested and confirmed by conventional PCR on various thermocyclers with equal reliability [MJR machine (MJ Research), Eppendorf, and Biometra]. The conditions were as described previously (9).

Among Greek-Cypriots, 2 frequent mutations are V726A and F479L. Together they account for ~50% of all MEFV chromosomes (9). Mutation F479L is either nonexistent or rare in all other populations tested (20). Surprisingly, F479L accounted for ~22% of the mutant alleles in our patient population, usually in compound heterozygosity with mutation V726A. Another interesting finding is that in the Greek-Cypriot population, F479L is always co-inherited in cis with variant E167D (allele 167D-479L), whereas in certain other populations, they were inherited separately. The derivative melting curves for mutation F479L demonstrated a clear difference of 10 °C between wild-type and mutant samples, leaving no chance for mistakes in results interpretation (see Fig. 1 of the online Data Supplement). Despite the presence of a prepeak in the homozygous mutant sample, this can never be confused with the profile obtained from the heterozygous sample, thereby ensuring a definite and safe diagnosis.

Mutation V726A is rather frequent in many populations tested, reaching 38% in Ashkenazi Jews, but is rare or even nonexistent in others, such as French and Spaniards (20). Among Greek-Cypriots, V726A accounts for ~28% of MEFV alleles. Using LightCycler technology and optimum FRET conditions, we designed the sequence of the detection probe to match the mutant amplicon perfectly (see Fig. 2 of the online Data Supplement). Derivative melting curves demonstrated a clear difference between the genotypes. The curve for the control showed a single wild-type peak, whereas samples from mutation carriers showed 2 different peaks, 1 for the wild-type allele and another 1 for the mutated allele. There was a large difference between the melting temperatures ($T_m$), nearly 9 °C; a prepeak shoulder in homozygous samples never interfered with interpretation.

We then attempted identification of mutations in the most commonly mutated region on exon 10, which spans amino acids 692–695 and harbors 5 of the most frequently occurring mutations in various populations (I692del, M694V, M694I, M694del, and K695R). The anchor and diagnostic probes were designed to encompass the relevant DNA sequence (Fig. 1). This combination was tested with samples bearing various combinations of genotypes as shown in Fig. 1B (individual melting curve plots are shown in Fig. 3 of the online Data Supplement and confirming agarose gels in Fig. 4 of the online Data Supplement). All samples were subjected to diagnosis after amplification with a common pair of PCR primers and the same combination of probes (Table 1 and Fig. 1A). Compared with the control, any sample containing 1 of the above 5 mutations had a lower melting profile, with the difference in $T_m$ varying from ~5 °C to >11 °C, permitting easy detection of mutations in either homozygosity or compound heterozygosity. As expected, the greatest difference was with the deletion mutations, which had melting profiles up to 11.1 °C (for mutation M694del) lower than the wild-type sequence. The least different but still distinctive melting profile was with the mutation K695R, which had a $T_m$ 5.2 °C lower than the wild-type sequence (Fig. 1B; see also Fig. 3 of the online Data Supplement). Discrimination among mutations was also possible based on the distinct melting profiles be-

![Fig. 1. Detection of 5 different mutations with a single probe in the region encompassing amino acids 690–696.](image-url)
cause of the high reproducibility of the $T_m$s. The samples with the closest $T_m$s were the substitutions M694V and K695R, which differed by 0.2 °C, a very narrow but still perceptible difference. Despite our confidence in discriminating the 2 profiles, it is prudent to corroborate such findings by alternative methods.

It is important to mention that the reproducibility of results in separate experiments and with unknown samples has been very high, with melting profiles for replicates of the same sample differing by <0.1 °C. The probes we designed successfully identified disparate mutations, including substitutions and small deletions, under a common set of conditions with a common pair of probes, based solely on the melting profile of the hybridization products. In case of uncertainty, however, an alternative approach can be used for further verification. A wild-type profile indicates with high certainty the absence of any of the 5 tested mutations and perhaps others that we did not examine in that same highly mutable region.

In summary, we present a robust, real-time PCR method using LightCycler technology for molecular investigation of patients referred for FMF. Until recently, the frequency of FMF in the Cypriot population of Greek origin was thought low; molecular genetic and epidemiologic investigations, however, have demonstrated the contrary. Recent results showed that island-wide, ~1 in 25 Greek-Cypriots is a carrier of a high-penetrance MEFV mutation. If the variant E148Q, which has lower penetration, is included, the frequency of carriers reaches 1 in 8. The low penetrance of E148Q is made evident by the fact that although it is very frequent in the general population, with a frequency of 1 in 12, only a few of our patient cohort carried it. We sought to use an approach that would allow easier and faster mutation analysis, with the idea that we could implement a national newborn-screening program, which would require such robustness.

To our knowledge, this method is the first to analyze MEFV mutations by FRET technology with a LightCycler. The possibility of carryover contamination, the nightmare of a routine clinical diagnostic laboratory, is drastically reduced because no post-PCR handling is necessary. In addition, nonspecific PCR products will not affect interpretation because the specific hybridization probes will not recognize them. Although this method requires special technology, it does not use hazardous material (ethidium bromide or acrylamide), is very rapid, and reduces labor and reagent costs. It takes advantage of the proximity of various mutations on exon 10, thereby simultaneously detecting any of the 5 mutations within the span of amino acids 692–695, and it identifies with certainty the exact defect in any combination.

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References

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