2004

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Recommended Citation

Dempsey, E., Barton, D., Ryan, F., : Detection of five common CFTR mutations by Rapid-Cycle Real Time amplification Refractory Mutation System PCR. Clinical Chemistry 50, No. 4, 2004. DOI: 10.1373/clinchem.2003.030445

Detection of Five Common *CFTR* Mutations by Rapid-Cycle Real-Time Amplification Refractory Mutation System PCR, *Eugene Dempsey,[1] David E. Barton,[2] and Fergus Ryan[1*]* ([1] Department of Biological Sciences, Dublin Institute of Technology, Dublin, Ireland; [2] National Centre for Medical Genetics and Department of Paediatrics, University College Dublin, Our Lady's Hospital for Sick Children, Crumlin, Dublin, Ireland; * address correspondence to this author at: Department of Biological Sciences, Dublin Institute of Technology, Kevin St., Dublin 8, Ireland; e-mail fergus.x.ryan@dit.ie)

Cystic fibrosis is the most common autosomal recessive disease in Caucasian populations and has a carrier frequency of 1 in 25 *(1)*. The gene involved codes for the cystic fibrosis transmembrane conductance regulator (CFTR), a membrane-associated protein involved in ion transport across the plasma membrane of epithelial cells. To date more than 1000 mutations have been described in this gene, and most are rare *(2)*. By focusing on five common mutations it is possible to detect the disease-causing mutation in ~90% of Irish patients *(3)*. The five mutations [and the percentages of Irish *(3)* and worldwide *(2)* cases] are F508del (77.4%, 66.0%), G551D (7.1%, 1.6%), R117H (2.7%, 0.3%), 621+1 G>T (1.4%, 0.7%), and G542X (0.5%, 2.4%). Four of these fall into the severe class of mutations in which the mRNA is incorrectly spliced (621+1 G>T), or in which the protein is not synthesized (G542X) or is blocked during processing (F508del), or its regulation is blocked (G551D).

Mutations in the *CFTR* gene can be detected by many mutation-detection systems, including single-strand conformation polymorphism analysis *(4)*, restriction fragment length polymorphism analysis, Amplification Refractory Mutation System (ARMS) PCR *(5)*, and more recently, real-time PCR systems using Sybr Green 1 *(6)* or hybridization probes *(7)*. Currently, for mutation detection using hybridization probes on the LightCycler system, the detector probe is designed to overlie the possible site of the mutation. This can have drawbacks when multiplexing in the same detection channel because peaks can become merged, making genotyping of a sample difficult.

This study aimed to improve the multiplexing capabilities of real-time PCR in its use for mutation detection. To this end we used ARMS PCR primers to selectively amplify the wild-type or mutant alleles in separate reactions. Subsequent detection of PCR products was carried out with a common set of hybridization probes. The advantage of this system is that it increases flexibility in design of the hybridization probe, allowing probes with precise melting temperatures (T_ms) to be generated. In this way melting curve peaks can be at a predetermined T_m for a particular PCR product, which allows for easier multiplexing of real-time PCR. Similar ARMS-based techniques have been developed for the ABI Prism 7700 sequence detector from PE Applied Biosystems using TaqMan® probes *(8)*. These protocols depend on the detection of an amplification curve and are limited to the detection of one mutation per color channel. Our protocol detects melting curve peaks post amplification and allows multiple mutations to be detected per color channel.

Both control samples for protocol optimization and test samples for the blinded study were obtained from the National Centre for Medical Genetics, Our Lady's Hospital for Sick Children (Crumlin, Dublin, Ireland). This study has received ethical approval from the DIT ethical review board (Project Ref. SF01/02).

Two multiplex PCR reactions were optimized by use of previously published ARMS primers *(5)*. The first reaction detects the G551D, R117H, and F508del mutations (87.2% frequency in Ireland, 67.9% worldwide). The F508del ARMS primers (CF-DFjN and CF-DFwM) are specific for this mutation and are not influenced by the benign I506V and F508C mutations. Because the CF-DFwM primer is complementary to the sequence distal to F508del, the presence of the I507del mutation is not recognized *(5)*.

The second reaction detects the 621+1 G>T and G542X mutations (1.9% frequency in Ireland, 3.1% worldwide). A PCR product should be formed in both the A and B ARMS reactions regardless of the sample genotype.

Three sets of fluorescence resonance energy transfer hybridization probes were designed to detect the 10 different ARMS PCR products. R117H and 621+1 G>T ARMS products are detectable by the CF4ARMS-A (5′-CCTTTTGTAGGAAGTCACCAAAGCAGTAC-F-3′) and CF4ARMS-P (5′-LCRed640-GCCTCTCTTACTGGGAA-GAATCA-P-3′) hybridization probes (F indicates a fluorescein, and P indicates phosphate). The F508del ARMS products are detected by the CF10ARMS-A (5′-GCA-CAGTGGAAGAATTTCATTCTGTTCTCAG-F-3′) and CF10ARMS-P (5′-LCRed640-TTCCTTGGATTATGCCT-P-3′) hybridization probes. The CF11ARMS-A (5′-TAT-GATTACATTAGAAGGAAGATGTGCCTTT-F-3′) and CF11ARMS-P (5′-LCRed705-AATTCAGATTGAGCAT-ACT-P-3′) hybridization probes detect both the G551D and G542X ARMS products. The predicted T_ms of the detector probes were calculated by use of MeltCalc software (www.meltcalc.com) *(9)*. All probes and primers were synthesized by Proligo, France.

ARMS PCR was carried out on a LightCycler (Roche Diagnostics). The total reaction volume was 10 μL, containing 1 μL of 10× LightCycler DNA Master Hybridization enzyme mixture (Roche Diagnostics), 3 mM $MgCl_2$, 0.1 μM hybridization probes, and ARMS primers concentrations as outlined in Table 1. ARMS PCR and subsequent melting curve analysis were carried out as follows:

Initial denaturation was for 7.5 min at 95 °C followed by 40 cycles of 95 °C for 1 s, 64 °C for 20 s, and 72 °C for 20 s. A touchdown PCR was carried out with the annealing temperature starting at 70 °C and decreasing to 64 °C at 1 °C/cycle. Fluorescence was measured at the end of the annealing step. The PCR stage was followed by a melting curve analysis (95 °C for 10 s, 40 °C for 30 s, and heating back up to 70 °C at 0.1 °C/s) with continuous monitoring of fluorescence. The touchdown

Table 1. Concentrations of *CFTR* ARMS primers for multiplex reactions.

ARMS primer name and sequence,[a] 5′–3′	Multiplex reaction	Primer concentration, ng/reaction
DF-C: ACTTCACTTCTAATGATGATTATGGGAGA	1A/B	100
DF-j-N: TATCTATATTCATCATAGGAAACACCACA	1A	100
DF-w-M: TATCTATATTCATCATAGGAAACACCATT	1B	100
11-C: TAAAATCAGCAATGTTGTTTTTGACCT	1A/B	150
GD-j-N: GCTAAAGAAATTCTTGCTCGTTGCC	1B	150
GD-j-W: AGCTAAAGAAATTCTTGCTCGTTGCT	1A	150
RH-C: CACATATGGTATGACCCTCTACATAAACTC	1A/B	120
RH-d-N: CCTCTGCCTAGATAAATCGCGATAGAAC	1A	120
RH-d-M: CCTCTGCCTAGATAAATCGCGATAGAAT	1B	120
621-C: TCACATATGGTATGACCCTCTATATAAACT	2A/B	200
621-j-N: TGCCATGGGGCCTGTGCAAGGAAGTATTCC	2A	200
621-j-M: TGCCATGGGGCCTGTGCAAGGAAGTATTCC	2B	200
11-C: TAAAATCAGCAATGTTGTTTTTGACCT	2A/B	40
GX-e-N: ACTCAGTGTGATTCCACCTTCTAC	2B	40
GX-e-M: CACTCAGTGTGATTCCACCTTCTCA	2A	40

[a] ARMS primer sequences are from Ferrie et al. *(5)*.

PCR conditions used allowed specific amplification of wild-type or mutated sequences as detected by standard agarose electrophoresis and melting curve analysis.

During the melting curve analysis, a temperature will be reached that causes the fluorescence resonance energy transfer to decrease as the detector probe is disassociated from its complementary sequence adjacent to the anchor probe. The first derivative of the melting curve is determined, which allows the decrease in fluorescence to be seen as a peak. When we used the MeltCalc software to assist in appropriate detector probe design, the actual T_ms seen during a melting curve analysis were within 1–2 °C of the predicted T_ms for the three detector probes. CF4ARMS-P, CF10ARMS-P, and CF11ARMS-P produced T_m peaks during melting curve analysis of ~62, 51, and 55 °C, respectively. In the melting curve profile of a representative sample shown in Fig. 1, the orientation of melting curve peaks can be clearly seen.

Using the optimized protocol as outlined in the methods, we blind-tested a panel of patient samples containing combinations of the five mutations. All patient samples were correctly identified and correlated with previous genotyping carried out by standard techniques.

The main advantage of the LightCycler system is its speed: the above protocols require ~ 45 min. Genotyping on the LightCycler system with standard multiplex hybridization probes that overlie the mutation site can be technically challenging and have some limitations. Even with careful design of the hybridization probes, wild-type and mutant alleles may not necessarily be differentiated from each other *(10)*. Our system allows hybridization probes to bind to any region of the amplicon, therefore providing scope for better probe design. The number of mutations that can be detected in a single reaction is dependent on the number of melting peaks that can be clearly differentiated from each other *(11)*. This usually leads to development of multiplex reactions in which a single mutation is detected in a color channel. Here we have demonstrated a method that makes it quite possible to detect more than one mutation in a single channel. By use of the nearest-neighbor formula in the MeltCalc software, detector probes can be carefully designed that

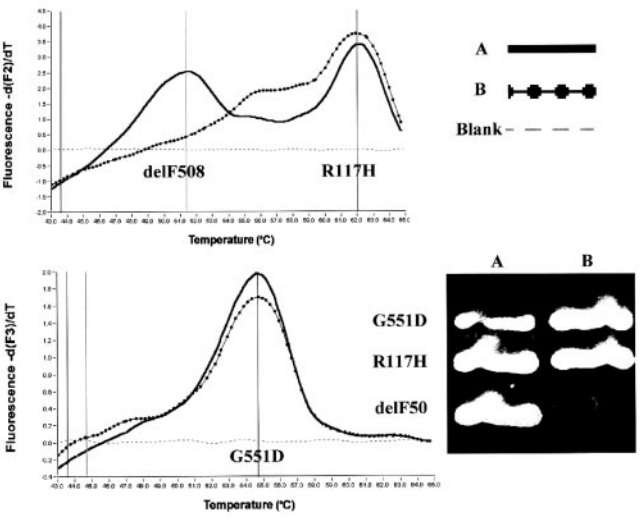

Fig. 1. Melting curve profile of a R117H/G551D compound heterozygote.

The F2 channel (*top plot*) detects both the wild-type and mutant F508del and R117H ARMS products with their respective peaks seen at ~51 and 62 °C. The F3 channel (*bottom plot*) detects the G551D ARMS products with a peak seen at 55 °C. *Reactions A* and *B* are set up as given in Table 1. The two peaks seen in the F2 channel at 62 °C and in the F3 channel at 55 °C show that this patient is a carrier for both mutations. A single peak at 51 °C in the F2 channel for the A reaction shows that only the wild-type F508del ARMS primers have produced a product for this sample. The agarose gel image also correlates with the melting curve peaks.

produce a peak within 1–2 °C of the predicted T_m. This allows T_m peaks, which indicate the presence or absence of a PCR product, to be strategically placed within the typical melting curve range of 45–70 °C for hybridization probes. Over this melting curve range we feel it should be possible to clearly distinguish three peaks in a single channel. This potentially makes it possible to multiplex six mutations in two capillaries when both fluorescent channels are used. This enhances the capabilities of the LightCycler for mutation detection; similar strategies could also be used for other real-time systems.

The results presented here demonstrate the ability to carry out multiplex mutation detection by use of a combination of ARMS PCR and real-time detection. A laboratory currently using ARMS PCR in a diagnostic setting can quite easily convert the standard ARMS PCR to a real-time ARMS PCR. This could have a major advantage in time savings and reduce the handling of potential carcinogenic ethidium bromide.

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DOI: 10.1373/clinchem.2003.030445

Improvement of Low-Density Microelectronic Array Technology to Characterize 14 Mutations/Single-Nucleotide Polymorphisms from Several Human Genes on a Large Scale, *Sabrina Frusconi,[1†*] Betti Giusti,[2†] Luciana Rossi,[2] Sara Bernabini,[1] Filippo Poggi,[2] Irene Giotti,[1] Rosanna Abbate,[2] Guglielmina Pepe,[2] and Francesca Torricelli[1]* ([1] Unita' Operativa Citogenetica e Genetica, Azienda Ospedaliera Careggi, Florence, Italy; [2] Dipartimento Area Critica Medico Chirurgica, University of Florence, Florence, Italy; † these authors contributed equally to this work; * address correspondence to this author at: Unita' Operativa Citogenetica e Genetica, Azienda Ospedaliera Careggi, Viale Morgagni 85, 50134 Florence, Italy; fax 39-055-4279686, e-mail genomica@ao-careggi.toscana.it)

Large-scale human genetic studies require new technologies to genotype several samples with relative ease, high accuracy, and reasonable costs. Among the available approaches, a microelectronic array technology has been developed for DNA hybridization analysis of mutations/single-nucleotide polymorphisms (SNPs) *(1–4)*. The microelectronic array system (NanoChip® Molecular Biology Workstation; Nanogen) produces a defined electric field that allows charged molecules, such as nucleic acids, to be transported to any test site, or pad, on the electronic chip (NanoChip cartridge). Electronic-based molecule addressing can rapidly achieve a high concentration of amplicons on each pad of the cartridge. Control of temperature allows use of an optimal thermal stringency to characterize a SNP/mutation in all 100 pads of a cartridge simultaneously *(5, 6)*. A thin hydrogel permeation layer overlies the pads; the presence of avidin or streptavidin in this layer allows the binding of biotinylated PCR products.

Although the technology is attractive, only a few protocols for its use have been published *(7–11)*. We describe the development, optimization, and validation of a high-throughput method for SNPs and mutations analysis that allows performance of 1372 characterizations on each chip.

We studied samples from 150 individuals for 14 SNPs/mutations previously characterized by standard methods (restriction analysis, automatic sequencing, and allelic discrimination). Genomic DNA was isolated from peripheral blood by use of the FlexiGene DNA reagent set (QIAGEN GmbH). We analyzed 14 DNA mutations/SNPs for a total of 2100 characterizations (homozygous wild type, n = 1367; heterozygous, n = 522; homozygous mutant, n = 211). The 14 nucleotide substitutions were SNPs/mutations involving the glycoprotein Ia (*GpIa*), glycoprotein IIIa (*GpIIIa*), follicle-stimulating hormone receptor (*FSHR*), hereditary hemochromatosis (*HFE*), and α_1 chain of collagen type 6 (*COL6A1*) genes.

For each mutation/SNP we designed a set of probes consisting of a forward and a reverse PCR oligonucleotide (one oligonucleotide for each SNP/mutation was 5′-biotinylated); two reporter oligonucleotides (one labeled with Cy3, specific for the wild-type nucleotide; the other, labeled with Cy5, specific for the mutant nucleotide), and