Technical Advance

Validation of High-Resolution DNA Melting Analysis for Mutation Scanning of the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) Gene

Marie-Pierre Audrezet,^{*†‡§} Aurélia Dabricot,[§]
Cédric Le Marechal,^{*†‡§} and Claude Ferec^{*†‡§}

From INSERM, U613,* Brest F-29200, France; the Faculté de Médecine et des Sciences de la Santé,[†] UMR-S613, University of Brest, Brest; Etablissement Français du Sang-Bretagne,[‡] Brest; Laboratoire de Génétique Moléculaire, Centre Hospitalier Universitaire de Brest, Hop Morvan,[§] Brest, France

High-resolution melting analysis of polymerase chain reaction products for mutation scanning, which began in the early 2000s, is based on monitoring of the fluorescence released during the melting of double-stranded DNA labeled with specifically developed saturation dye, such as LC-Green. We report here the validation of this method to scan 98% of the coding sequence of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. We designed 32 pairs of primers to amplify and analyze the 27 exons of the gene. Thanks to the addition of a small GC-clamp at the 5' ends of the primers, one single melting domain and one identical annealing temperature were obtained to co-amplify all of the fragments. A total of 307 DNA samples, extracted by the salt precipitation method, carrying 221 mutations and 21 polymorphisms, plus 20 control samples free from variations (confirmed by denaturing high-performance liquid chromatography analysis), was used. With the conditions described in this study, 100% of samples that carry heterozygous mutations and 60% of those with homozygous mutations were identified. The study of a cohort of 136 idiopathic chronic pancreatitis patients enabled us to prospectively evaluate this technique. Thus, high-resolution melting analysis is a robust and sensitive single-tube technique for screening mutations in a gene and promises to become the gold standard over denaturing high-performance liquid chromatography, particularly for highly mutated genes such as *CFTR*, and appears suitable for use in reference diagnostic laboratories. (*J Mol Diagn* 2008, 10:424–434; DOI: 10.2353/jmoldx.2008.080056)

The gene responsible for cystic fibrosis (CF), called the *CFTR* gene, was cloned nearly 20 years ago thanks to a successful positional cloning strategy.^{1–3} This was the starting point of an intensive collaborative world-wide project performed by the Cystic Fibrosis Genetic Analysis Consortium (<http://genet.sickkids.on.ca>). *CFTR* is one of the most highly mutated human genes with more than 1500 different mutated alleles reported so far. Besides the most frequent mutation, a 3-bp deletion, the F508del, 30 to 50 mutations are commonly found in different countries but their distribution varies according to the ethnic or geographic origin of CF patients.⁴

Most of the 1500 mutated alleles are extremely rare and many of them are private mutations. It was rapidly evident to us and others that in such a situation, the best strategy to identify these mutations was to use a scanning technique to analyze the coding sequence of the gene rapidly. In the 1990s, we were the first to report, by using the denaturing gradient gel electrophoresis technique, that nearly all of the mutated alleles could be identified in a population such as that of Brittany.^{5,6}

As an alternative and less-time consuming technique, we proposed in the 2000s that the gold standard for fully analyzing the coding sequence of the *CFTR* gene was the denaturing high-performance liquid chromatography (DHPLC) technique followed when necessary by direct sequencing. We reported detection of nearly 100% of point mutations, and this remains true 8 years later.⁷ However, a new scanning technique for heterozygote

Supported by the INSERM (Institut National de la Santé et de la Recherche Médicale), the PICRI Project (Partenariats institutions-citoyens pour la Recherche et l'Innovation), the French Association Vaincre La Mucoviscidose, the Association de Transfusion Sanguine et de Biogénétique Gaëtan Saleun, and the Programme Hospitalier de Recherche Clinique R08-04 and 'Evolution de l'épidémiologie Génétique de la Mucoviscidose dans le Grand Ouest de la France.

Accepted for publication May 27, 2008.

Supplemental material for this article can be found on <http://jmd.amjpathol.org>.

Address reprint requests to Marie-Pierre Audrezet, Laboratoire de Génétique Moléculaire, CHU, 29200 BREST, France. E-mail: marie-pierre.audrezet@univ-brest.fr.

detection, called high-resolution melt curve analysis (HRM), appeared more recently. The melting curve analysis in conjunction with real time polymerase chain reaction (PCR) was first proposed in 1997,^{8,9} and the introduction of a new family of LC-Green dyes allowed accurate SNP genotyping and heterozygote scanning in small amplicons.^{10,11} Heterozygous samples are identified by differences in melting curve shape. When a nucleotide change is present in a target sequence the resulting melting curve of the PCR product is a composite of both heteroduplex and homoduplex components that is visualized by the different melting curve shapes of the two species present. HRM is a closed-tube method that uses standard PCR primers and requires no post-PCR processing. Because this method seems rapid, robust, and less expensive than other scanning techniques, we decided to analyze the *CFTR* gene by HRM, to define our conditions according to the melting curve profile of the coding sequence of the 27 exons of the gene and to compare the results with our DHPLC technique that has been routinely used for 8 years in our laboratory. Our results and HRM conditions are reported together with the excellent sensitivity achieved for heterozygote detection (consistently 100%). In the case of the scanning of CF patients, who can be homozygous, analyzing the sample mixed with a known wild-type DNA allows identification of a mutation at a homozygous status. So, HRM is a robust rapid and very promising technique for scanning the *CFTR* gene for routine diagnostic investigation as well as for research projects.

Materials and Methods

DNA

DNA samples ($n = 307$), extracted by the salt precipitation method, carrying 221 mutations and 21 polymorphisms, 20 control samples free of variations (confirmed by DHPLC analysis), and 136 samples of patients with idiopathic chronic pancreatitis (ICP) were used in this study. The list of the mutations and SNPs is given in supplemental Table 1 (available at <http://jmd.amjpathol.org>). A written consent was obtained from each patient included in this study.

Primer Design

New pairs of primers were designed using PrimerQuest software (Integrated DNA Technology, Coralville, IA) to amplify the coding sequences and the intron-exon junctions (4 to 87 bp each side) (Table 1). The melting curves of the exons were analyzed with Navigator Software (Transgenomic Inc, Omaha, NE). An artificial GC clamp was added to the 5' ends to obtain a single melting domain for each fragment and to increase the T_m of the primers to allow amplification under identical conditions for all amplicons. Each exon was analyzed in a single PCR product, except exons 13 and 17b, which were split into five and two amplicons, respectively. Specific forward and reverse primers for exon 9 were designed after alignment with sequences of high homology. The polymorphic sequences of exons 6b (GATT) and 9 (TG and T), and the 5'

part of exon 10 containing the frequent benign variant M470V, were excluded.

DNA Amplification

PCR was performed in a 10- μ l reaction mixture containing 4 μ l of LightScanner Master Mix (Idaho Technology Inc., Salt Lake City, UT), 0.3 μ mol/L of each primer, and 10 ng of DNA. Cycling conditions consisted of an initial denaturation step at 94°C for 2 minutes, followed by 45 cycles of 30 seconds at 94°C and 30 seconds at 62°C, and a final cycle of 30 seconds at 94°C and 30 seconds at 25°C. Amplicons were stored in the dark at 4°C before melting. PCR efficiency, specificity, and size of the amplicons were checked by agarose gel electrophoresis and analysis of derivative melting curves on LightScanner Software with Call-IT v1.5 (Idaho Technology Inc.).

Melting Acquisition

After amplification, samples can be kept in the dark up to 1 week until melting analysis. PCR products are melted by increasing the temperature from 60 to 98°C at a programmed rate of 0.1°C/second with exposure of 14 to 30 milliseconds.

HRM Optimization

Six wild-type samples and two to twenty DNA samples carrying mutations located in the fragments were used as negative and positive controls. HRM analysis was performed according to the manufacturer's recommendations with LightScanner Software with Call-IT v1.5 (Idaho Technology Inc.).

Data Analysis for ICP Patients

Results were blindly interpreted by two researchers. Six wild-type samples and three positive controls carrying the mutations most difficult to identify were used for the analysis of samples from ICP patients.

Direct Sequencing

Samples from ICP patients showing abnormal HRM profiles were sequenced with the BigDye Terminator cycle sequencing kit v1 (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations and analyzed on capillary electrophoresis with ABIPrism 3130 (Applied Biosystems). Sequences of the primers used for sequencing are given in Table 2.

Results

Primer Design and PCR Optimization

PCR specificity is an indispensable prerequisite for HRM curves analysis. Primers have been designed to amplify almost the whole coding sequence and the intron-exon junctions (4 to 87 bp), taking into account the presence of intronic mutations to be analyzed and intronic polymor-

Table 1. Sequences of the Primers Used for CFTR Analysis by HRM, GC Size, Amplicon Length, Number of Positive Controls Validated for Each Exon, and Positive Controls for Routine Analysis

Exon	Primer	Sequences	GC length	Amplicon length (bp)	Introns	Number of heterozygous-positive controls	Number of homozygous-positive controls	Recommended control
1	LSCFE1Fmod	5'-CCGCCGCCGTTGAGCGGCAGGCACC-3'	8	200 bp	74	4		125G>C M1I
	LSCFE1Rmod	5'-CCGCCGCCGCGCACGTGTCTTT CCGAAGCT-3'	8		19			
2	2i5b	5'-CAAATCTGTATGGAGACC-3'	0	194 bp	39	5		R31C 296+1G>T
	2i3"	5'-CAACTAAACAATGTACATGAAC-3'	0		4			
3	LSCFE3Fmod	5'-CGCCGTTAAGGGAAATAGGACAA CTAAAATA-3'	5	276 bp	44	10	2	R75Q
	LSCFE3Rmod	5'-CCGCCGATTACACAGATTCGTAGTC-3'	6		66			
4	LSCFE4FmodC	5'-CCGCCGCCGCCCGTGTGAAATT CTCAGGGT-3'	12	361 bp	52	14	1	G85V R117H
	LSCFE4RmodC	5'-CCGCCGCCCACATGTACGATAC AGAATATATGTGCC-3'	9		26			
5	LSCFE5Fmod	5'-CCGCCGGTTGAAATTATCTAAGTTTC-3'	6	201 bp	13	8		624delT 711+1G>T
	LSCFE5Rmod	5'-CCGAAGTCCGCTTCCAGTTGT-3'	3		48			
6a	LSCF6aFmod2	5'-CCGCCGGGGTGGAAAGAT ACAATGACACCTG-3'	5	317 bp	25	8		C225X
	LSCF6aRmod2	5'-CCGCCGCCGCGATGCATAGAG CAGTCCTGGTT-3'	11		66			
6b	LSCFE6bFmod	5'-CGCGCCGCCGATTAC AGAGATCAGAGAG-3'	10	239 bp	0	2	1	R258G
	LSCFE6bRmod	5'-CCGCCGCCGAGGTGGA GTCTACCATGA-3'	8		66			
7	LSCFE7Fmod2	5'-CCGCCGCCCTCTCCCTGAATTT TATTGTTATTGTTT-3'	13	326 bp	7	11		1078delT
	LSCFE7Rmod2	5'-CCGCCGCCCTATAATGCAG CATTATGGT-3'	10		7			
8	LSCFE8Fmod	5'-CCGGAATGCATTAATGCTAT TCTGATTC-3'	4	199 bp	32	7		W401X
	LSCFE8Rmod	5'-CCGCAGTATAGGTGTTAG AGCAAAACA-3'	4		18			
9	LSCFE9Fmod2	5'-CCGCCGCCGGAATTATTGAGAA AGCAAAACA-3'	8	279 bp	0	3		D443Y
	LSCFE9Rmod2	5'-CCGCCGCCGAAAATACCTTCCAG CACTACAACTAGAAA-3'	8		57			
10	LSCF10FmodD	5'-CGCGTTATGGGAGAACTGG AGCCTTCAGAG-3'	5	275 bp	0	15	1	F508del
	LSCF10RmodD	5'-CCGCAGACTAACCATTGAAT ATGGAGCC-3'	4		68			
11	h11i5	5'-TGCTTTTCAAATTCAGATTGAGC-3'	0	197 bp	42	13	2	G542X G551D
	11i3ter	5'-ACAGCAAATGCTTAGACC-3'	0		17			
12	LSCFE12Fmod	5'-CGCGTCATCTACACTAGATGACCAG-3'	4	244 bp	43	15		G576A
	LSCFE12Rmod	5'-CCGAGGTAAATGCAATCTATGATG-3'	3		63			
13	LSCF13AFmod	5'-CCGCCGCCGGAGACATATTG CAATAAAGTAT-3'	9	346 bp	38	20		1898 + 1G>A I601F
	LSCF13ARmod	5'-GCCTGTCCAGGAGACAGGA GCATCTC-3'	2					
13	LSCF13BFmod	5'-CCGCCGCAATCCTAAGTACGAG ACCTTACACCG-3'	2	346 bp				R668C
	LSCF13BRmod	5'-CCGCCGATCAGGTTCAGGA CAGACTGC-3'	3					
13	LSCF13CFmod	5'-CCGCGGTGATCAGCACTGGCCC-3'	6	301 bp	77			L749L V754M
	LSCF13CRmod	5'-CCGCGCGCGCGCCAGTTCTTG AGATAACCTTCT-3'	13					
13	LSCF13DFmod	5'-CGTGTCACTGGCCCCTCAGGC-3'	1	221 bp				I807M
	LSCF13DRmod	5'-CCGCCGCCGCTAATCCTATGA TTTTAGTAAAT-3'	9					
13	LSCF13FFmod	5'-CGCGGTGCAGAAAGAAGAAAT TCAATCCTAAGT-3'	4	220 bp				R668C
	LSCF13FRmod	5'-CCGCCGTGCCATTTCATTTGT AAGGGAGTCT-3'	6					
14a	LSCF14aFmodB	5'-CCGACCACAATGGTGGCAT GAAACTG-3'	3	239 bp	35	7	1	T854T
	LSCF14aRmodB	5'-CCGCCGACTTTAAATCCAGTAAT ACTTTACAATAGAACA-3'	6		7			
14b	LSCF14bFmod	5'-CCGAGGGAATAGGTGAAGAT-3'	2	179 bp	38	4		W846X 2752-5G>T
	LSCF14bRmodb	5'-CCGTACATACAAACATAGTGATT-3'	3		59			
15	LSCFE15Fmod	5'-CGCGCCGTGTATTGGAAA TTCAGTAAGTAACCTTGG-3'	7	412 bp	33	16		2789+5G>T T908S
	LSCFE15Rmod	5'-CCGCAGCCAGCACTGCCAT TAGAAA-3'	4		68			

(table continues)

Table 1. *Continued*

Exon	Primer	Sequences	GC length	Amplicon length (bp)	Introns	Number of heterozygous-positive controls	Number of homozygous-positive controls	Recommended control
16	LSCFE16Fmod	5'-CCGCTGAATGCGTCTACTGTGATCCA-3'	3	299 bp	77	6		G970R
	LSCFE16Rmod	5'-CCGTAGACAGGACTTCAA CCCTCAATCAA-3'	3		87			3120+1G>A
17a	LSCFE17AFmod	5'-CCGCCGGACACTTTG TCCACTT-3'	6	286 bp	49	13		3121-1G>A
	LSCFE17ARmod	5'-CCGCCGTCAAATAGCTCTTATAGCTTTTT ACAAGATG-3'	6		25			I1027T
17b	LSCF17BAFmod	5'-CCGCCGCCCGCCGTCAGGTACA AGATATTATG-3'	14	247 bp	56	11		3272-26A>G
	LSCF17BARmod	5'-CCGCCGCCCGCAGTGTGACAGGT ACAAGAAC-3'	7					A1067T
	LSCF17BBFmod	5'-CCGCCCTTACTTTGAACTCTGTT CCACAAAGC-3'	4					T1095T
	LSCF17BBRmod	5'-CCGCCGTTGATAACCTATAGAATG CAG-3'	6		62			E1104X
18	LSCFE18Fmod	5'-CCGCCGAGTCGTTACAGAAGA GAGAAATAAC-3'	6	236 bp	34	2		D1152H
	LSCFE18Rmod	5'-CCGCCGCCCGCGGTACTTTGTT ACTTGTCTGAATTTTTT-3'CATAA	12		25			3547delA
19	LSCF19i5mod	5'-CCGCCGCCCGCGCATCAAATA ATTGTGAAATTGTCTGCC-3'	10	408 bp	73	10		S1235R
	LSCF19i3mod	5'-CCGCCGCCCGCACATTGCT TCAGGCTACTGGGA-3'	11		49			R1162L
20	LSCF20i5mod	5'-CCGCCGCCCGCGCTACTGAATTATGT TTATGGCATGG-3'	13	323 bp	44	13		W1282X
	LSCF20i3mod	5'-CCGCCGCCGCTCTTGAGTACAAGTA TCAAATAGCAG-3'	10		50			4005+33G>A
21	LSCFe21F	5'-CCGCCGCCCGCGCAAGTTATTCATA CTTTCTTTCTTT-3'	12	217 bp	15	5	1	N1303K
	LSCFe21R	5'-CCGCCGCCGCTATATCAGCCA TTTGTG-3'	8		47			Q1313X
22	LSCFe22FmodC	5'-CCGCCGAGAATGTCAAC TGCTTGAGTGT-3'	6	311 bp	41	2		R1358S
	LSCFe22RmodD	5'-CCGCCGGCAGGCATAATGA TTCTGTTCCAC-3'	10		51			I1366T
23	LSCFE23Fmod	5'-CCGCCGCCGCAAGGTAAAT ACAGATCAT-3'	9	259 bp	44	3		4374+1G>T
	LSCFE23Rmod	5'-CCGGCAGGAATCATCAT GTGAGATTG-3'	3		53			4374+13A>G
24	LSCFE24FmodB	5'-CCGCCGCTTTGAGCCTGT GCCAGTTTCTGT-3'	6	378 bp	58	5	1	Q1463Q
	LSE24RmodB	5'-CCGCCGACGAGCTCCAATTC CATGAGGTGA-3'	6		62			Y1424Y

phisms that we have chosen to exclude. For example, the frequency of the mutation 3272-26A>G in intron 17a required the forward primer to be located 56 bp upstream. Conversely, forward primers for exons 6b, 9, and 10 were located in the exons to avoid the highly polymorphic poly (GATT), poly (TG), and poly (T) tracts as well as the frequent polymorphism M470V. This represents 89 bp of 4443 bp, which corresponds to 19 known mutations of 1557. Specificity of the primers was checked in University of California, Santa Cruz *In Silico* PCR.

Melting curves of the fragments were analyzed using Navigator Software to assess the number of melting domains of the amplicons, which varied from two to five according to the exon, prior reduction by addition of an artificial GC clamp of 2 to 14 bp (Figure 1 and Table 2). Annealing temperature gradient from 56 to 63°C in the presence of LightScanner MasterMix (Idaho Technology Inc.) was used to determine the annealing temperature in which specific PCR products were obtained. Where frag-

ments showed only one melting domain, an artificial GC clamp seemed unnecessary, but PCR efficiency was unsatisfactory at 62°C, the best temperature for the majority of amplicons. Because uniform PCR conditions are better for routine use, the primers of exons 3, 5, and 15 were modified with a GC clamp of 3 to 7 bp, to obtain specific amplifications at 62°C.

HRM Analysis

The same six wild-type samples were used throughout as negative controls, to ensure that the same normal profile was reproducibly obtained. The influence of DNA quality was tested on wild-type samples, especially DNA extracted from blood spots. Unfortunately, DNA quality is a critical parameter for analysis by HRM, and the results obtained with DNA extracted from the Guthrie card were uninterpretable. Samples should be homogeneous and extracted by

Table 2. Sequences of the Primers Used for Sequencing

Exon	Primer	
1	CF1Fseq	5'-GGAAGCAAATGACATCACAGCAG-3'
	CF1Rseq	5'-ATACACACGCCCTCCTCTTTTCGT-3'
2	CF2Fseq	5'-CGCGTTTCCATATGCCAGAAA-3'
	CF2Rseq	5'-CGCCGTAATATGAATTTCTCTCTCAACT-3'
3	CF3Fseq	5'-CGCTCCTTGGATATACTTGTGTGAATC-3'
	CF3Rseq	5'-CGTGTTTGGAGTTGGATTCATCCTT-3'
4	CF4Fseq	5'-CCGAATAGTGCTAAGAGTTTCACATATGGTA-3'
	CF4Rseq	5'-CCGAATTAATTTTCAGCATTTATCCCTTACT-3'
5	CF5Fseq	5'-CGCCGACCTTTACTTAATAATGAATGC-3'
	CF5Rseq	5'-CGCGTGAGCAAGACTTAACCACTA-3'
6a	CF6aFseq	5'-GTGTGCTCAGAACCACGAAGTGT-3'
	CF6aRseq	5'-CGGACACTGAAGATCACTGTTCTATGC-3'
6b	CF6bFseq	5'-CGCCGTTGAGCAGTTCTTAATAGATAA-3'
	CF6bRseq	5'-CCGGAGGTGGAAGTCTACCATGA-3'
7	CF7Fseq	5'-CGCACAGGTCAATATGATGTGGAG-3'
	CF7Rseq	5'-CGCGATTAGAACTGATCTATTGACTGAT-3'
8	CF8Fseq	5'-CGCGAGATGTAGCACAAATGAGAGT-3'
	CF8Rseq	5'-CCGCAGTTCTACCACTTATATCATCATTC-3'
9	CF9Fseq	5'-AATTATCTGGCAATCTCTTCAT-3'
	CF9Rseq	5'-TATTATACAAGACACTACCCATACA-3'
10	CF10Fseq	5'-CCGGCAGAGTACCTGAAACAGGA-3'
	CF10Rseq	5'-CCGCATTACAGTAGCTTACCA-3'
11	CF11Fseq	5'-GCATTTGAAATTAATGGAGATGCAATGTTC-3'
	CF11Rseq	5'-TGACACCAAGATACGGGCACAGAT-3'
12	CF12Fseq	5'-CGGTGCTACTTCTGCCCACTT-3'
	CF12Rseq	5'-GAAACTGGTTTAGCATGAGGCGGT-3'
13	CF13Fseq	5'-CATGTTATTTTCATGCTATCAG-3'
	CF13Rseq	5'-ACTAAATCCTTTCCCATTT-3'
14a	CF14aFseq	5'-CGCGTGACATCATCATGGC-3'
	CF14aRseq	5'-CGCGCTAAGAAGTATATGAATCTATACCA-3'
14b	CF14aFseq	5'-GGGAGGAATTAGGTGAAGAT-3'
	CF14aRseq	5'-TACATACAAACATAGTGGATT-3'
15	CF15Fseq	5'-CGGTGCATGCTCTTCTAATGCAA-3'
	CF15Rseq	5'-GGAGGTTCAACAAGGGCACAT-3'
16	CF16Fseq	5'-CCGAGAATGTTTGGAAAGAAACAA-3'
	CF16Rseq	5'-CCGCACTCCCAAGTAGACAGCTA-3'
17a	CF17aFseq	5'-GCACCAGCATGGCACATGTAT-3'
	CF17aRseq	5'-CCGCCATGTGTACTTTGTAATATAGTTTCC-3'
17b	CF17bFseq	5'-GTTCCTCTCTCTGGTAGCCAAGTA-3'
	CF17fRseq	5'-TGGAAATTCAAAGAAATCACTTGTTC-3'
18	CF18Fseq	5'-CATGTGCCCTAGGAGAAGTGTGA-3'
	CF18Rseq	5'-CACAGGTGAAAGAATGCTCACTGC-3'
19	CF18Fseq	5'-CGCGCAATTATTTCTGTTAGTTC-3'
	CF18Rseq	5'-CGCGTCTGCAAGTACAATCACA-3'
20	CF20Fseq	5'-CGGGTCAGGATTGAAAGTGTGC-3'
	CF20Rseq	5'-GCTATGAACATTTCAATAACCAGAATTGAG-3'
21	CF21Fseq	5'-CGAAGAATGATACAAAGCAGACATGA-3'
	CF21Rseq	5'-CGTGTCTCTCCAGGTATGTTAGGG-3'
22	CF22Fseq	5'-CCGTGAGCTGTCAAGTTGTAAATAGACT-3'
	CF22Rseq	5'-CACACGCAGACATGACAGCCTAATA-3'
23	CF23Fseq	5'-CGCGATAGAACAATAGACATATTATCAAGG-3'
	CF23Rseq	5'-GCTGCACAAGTTTTCGCG-3'
24	CF24Fseq	5'-CCGTGTGACATACCTGATTGTTCA-3'
	CF24Rseq	5'-CCGCGCTTAATTCATCCTTGTTT-3'

the same technique: the majority of our samples were extracted by a classical saline technique or an automated extraction and their quality was adequate. We had in our DNA bank, 307 samples carrying different mutations and polymorphisms that correspond to 2 to 20 positive samples per exon. We used them to validate the analytical conditions and possibly to modify our primer positions.

We have thus been able to verify the sensitivity of the technique, because all of the 232 heterozygous variants were detected. Moreover, different melting profiles were reproducibly obtained for samples carrying different variants (Figure 1)

and reproducible melting profiles were obtained with different samples carrying the same mutation, or mutations located in the same codon (Figure 2; exon 21, mutation N1303K, T1299T). However, even if the technique is reproducible enough and if a particular melting curve is generally associated with a particular mutation, each positive amplicon still must be sequenced to absolutely determine what mutation is present.

Certain exons of the *CFTR* gene contain frequent polymorphisms, such as T854T in exon 14a, which could have concealed a mutation in cis or in trans. Figure 3 shows the

Figure 1. Melting profiles of different mutations of exon 8: 1249-5A>G, L375F, 1259insA, T388M, and W401X.

difference plot of wild-type samples, the polymorphism T854T alone or combined with another rare variant, I853I. This provided the opportunity to check for concealment of mutations within the polymorphism. Two different mutations may also be present on the same exon. Different samples, compound heterozygous have been tested and we have been able to confirm that an additional variant modifies the melting curve, compared to those of a DNA heterozygous for a single mutation (Figure 4).

In addition to heterozygous variants, 10 homozygote samples carrying F508del, 394delT, R117H, G542X, S549R, 4016inT homozygous mutations, and R75Q, T854T, 1001+11 C>T, and Q1463Q homozygous variants, were also tested. Only mutations 394delT, R117H, G542X, 4016insT, and variants R75Q and 1001+11 C>T provided positive results (Figure 5). Samples carrying mutations in the homozygous state are rare, but the most frequent mutation, F508del, which accounts for ~70% of CF alleles and is present in homozygous state in approximately half of the patients, was not detected. We detected 60% of the homozygous mutations tested. So, in the case of the scanning of a CF child for example, if the DNA sample can be homozygous, it is necessary to work additionally on at least one parental sample, or to genotype the sample by mixing

Figure 2. Difference plot of samples carrying five different variants in exon 21. Different melting profiles are obtained for samples carrying different variants and reproducible melting profiles are obtained with different samples carrying the same mutation, or mutations located in the same codon.

Figure 3. HRM analysis data for exon 14a, which contains the frequent polymorphism T854T. The figure shows that the difference plot profile is modified when another variant (I853I) is associated with this frequent polymorphism.

it with a known wild-type sample. Figure 6 illustrates the difference plots obtained for a sample homozygous for F508del mutation with or without mixing with a normal DNA before PCR (Figure 6a), and after a first melting (Figure 6, b). The samples may be first analyzed without mixing, and when the result is negative for DNA from CF patients, the PCR product can be mixed with that of a normal DNA. After a denaturation/hybridization cycle of heating at 94°C followed by a rapid cooling to 45°C to generate heteroduplexes, a melting profile corresponding with those of a heterozygous sample is obtained.

The large number of mutations tested enabled us to produce a large panel of difference plot profiles, and therefore to determine the variants of each exon that are difficult to identify, and to select ideal routine controls. For example, the 1078delT mutation, which is frequent in Brittany has a subtle curve modification, requiring increased sensitivity of the analysis parameter, and we chose it as a control for the systematic analysis of exon 7 (Figure 7). Because of their size and of the number of melting domains, exons 13 and 17b were split into five and two fragments, respectively. Figure 8 shows the profiles obtained for exon 17b.

Exon 13 was initially split into four overlapping fragments and we had 20 different positive controls distributed throughout the exon. First analysis indicated that the region between codons 670 and 709 (nucleotides 2140 to 2259),

Figure 4. HRM analysis data for exon 10, which contains the frequent mutation F508del. Differences in melting plots obtained for a heterozygous F508del and two compounds heterozygous (F508del/S492F and F508del/S489X).

Figure 5. Some homozygous variants detected: 394delT in exon 3, 1001+11C>T in exon 6b, G542X in exon 11, and 4016insT in exon 21.

which included a polyA-repeat was not correctly processed, probably because of the presence of two melting domains in this fragment. We have therefore developed a fifth fragment covering this region that was finally validated.

Validation of the Technique Using Samples from 136 Patients with ICP

A part of ICP is considered as a *CFTR*-related disorder. In young patients, when all other etiologies have been discarded, in ~30 to 40% of patients, a mutated *CFTR* allele is commonly reported in the literature.^{12–14} Once conditions of analysis were established for each exon, we studied a cohort of 136 patients with ICP. Our objective was to prospectively evaluate the technique, particularly the false-positive and potential false-negative rates. After a double-blind interpretation, the samples were classified into negative, positive, or doubtful for the two samples farthest from the baseline. All positive or doubtful samples were sequenced. A mutation or a polymorphism was identified in all of the positive-classified samples whereas negative sequencing results were obtained for the questionable samples. Twenty-eight different mutations and twenty-two different polymorphisms have been identified in this series (Table 3), including fifteen variants that were not among our positive controls. This brings the number of *CFTR* variants validated by the technique to 247. Moreover, samples carrying abnormalities were identified in all

exons except exons 8, 14b, 16, and 22. In this series of 136 patients, 33 carried at least one *CFTR* mutation and 8 of them were compound heterozygous for two mutations. These results are consistent with previous data published in the literature.

Discussion

Several mutation scanning methods have been developed in recent years, including single strand conformation polymorphism,¹⁵ DGGE,¹⁶ and DHPLC.^{17,18} All of these methods require the separation of the samples on a gel (for single strand conformation polymorphism and denaturing gradient gel electrophoresis) or on a column (for DHPLC), remain demanding, manual, and time-consuming, and sometimes require sophisticated and expensive instrumentation.

HRM analysis for mutation scanning began in the early 2000s. It is based on the monitoring of the fluorescence released during the melting of a double-strand DNA labeled with a new generation of saturation dyes, specifically developed for HRM, LC-Green. Initially developed on existing real-time PCR thermocyclers, improvement of the technique rapidly necessitated specific instruments and software, and to date, nine different platforms, which have been the subject of various evaluations are available.^{19–21}

The increasing number of publications reflects the interest of laboratories in this new technology, which has

Figure 6. Analysis of a homozygous F508del sample. Difference plots obtained with or without mixing with a normal DNA before PCR (**a**) and after a first melting (**b**). **a:** DNA from a CF patient with analyses alone or after mixing with a known wild-type sample before amplification. The profile obtained for the mixed DNA corresponds to those of a heterozygous sample. **b:** The samples may also be first analyzed without mixing, and when the result is negative for DNA from CF patients, the PCR product can be mixed with that of a normal DNA. After a denaturation/hybridization cycle of heating at 94°C followed by a rapid cooling to 45°C to generate heteroduplexes, a melting profile corresponding with those of a heterozygous sample is obtained.

already been successfully applied to the analysis of *TP53*,²² *phenylalanine hydroxylase* gene,²³ factor VIII,²⁴ factor II, factor V, *HFE*,^{11,25,26} *C-kit*,²⁷ *EGFR* *HER2*,²⁸ *RET*,²⁹ and *CFTR*.³⁰ In this last study, the authors report the *CFTR* scanning by HRM after PCR amplification of 37 exon/intron fragments in 2 panels of 96 random white UK blood donors and 30 blinded DNA samples enriched for

Figure 7. Detection of three common mutations in exon 7. The 1078delT mutation, which is frequent in Brittany shows a subtle curve modification and must be included as a control for the systematic analysis of exon 7.

CF-causing variants. They were able to identify 22 variants in the random panel and 31 mutations in the 30 blinded panel. However, the variants identified in this study were located in 15 exons of 27 and only 1 variant was observed in 12 exons.

The sensitivity of this technique was compared with that of DHPLC, currently the most widely used technique for the analysis of genes such as *CFTR*.³¹ This limited study analyzed 22 samples containing 16 mutations located in six exons, using both techniques, and sensitivity of HRM analysis appeared superior to that of DHPLC. However, under the preferred DHPLC conditions, the frequent mutation R117H of exon 4 was not detected, and the percentage of false-positive results was significant, suggesting that analytical conditions were not fully optimized.

We have also developed DHPLC for *CFTR* analysis.⁷ Twenty-seven exons of the *CFTR* gene were studied from 415 samples of different carriers, polymorphisms, and mutations. In our experience, once conditions of analysis for the different areas of fusion molecules are validated, the sensitivity of this technique is 100%. In this work, we tested 415 DNA samples but unfortunately now approximately half of these samples have been used as controls for many years and were depleted and not available for the present study.

We chose to develop HRM for rapid screening of 98% of the coding sequence of the *CFTR* gene and to obtain a sensitivity that would be at least as good as DHPLC, our gold standard technique. Exons of the *CFTR* gene vary between 38 and 251 bp (with the exception of exon 13 that is 784 bp long and needs to be cut up into several fragments), which is consistent with an analysis by HRM providing good specificity and sensitivity. Dependence of sensitivity and specificity on size and type of base change has been demonstrated by Reed and Wittwer,³² who recommend using PCR products of 300 bp or less.

Our objectives were to obtain one single melting domain and one identical annealing temperature to co-amplify all of the fragments. Experience with techniques such as denaturing gradient gel electrophoresis and DHPLC informed our analysis of melting curves of DNA fragments. Regarding DHPLC, the presence of different domains sometimes required multiple conditions for analysis. For HRM, mutation detection is easier when there is a single melting domain because the presence of many domains could require cutting into several fragments, thus increasing the number of amplifications and raising the cost of the analysis. We anticipated that adding a small artificial GC clamp at the 5' ends of primers could in some cases smooth melting curves to reduce the number of melting domains. This has been achieved for all exons with the exception of exon 3 and part of exon 13B. For exon 3, the presence of two domains did not affect the results because 12 positive controls located in the two domains showed positive profiles. For the second part of exon 13 (named 13B) the 5' section of the fragment corresponding to the first break in the slope of the melting curve, with the lower melting temperature not adequately analyzed, and we therefore designed a fifth pair of primers. So, in this work we demonstrated that the sensitivity of the technique is not size-dependent but melting domain-dependent.

In the study of Krypuy and colleagues²² on *TP53*, the authors also report the use of modified primers (without

Figure 8. HRM analysis data for exon 17a. Because of its size and of the presence of many melting domains, it was split into two overlapping fragments. **Part A** contains codons 1047 to 1088 and **part B** contains codons 1082 to 1122.

specifying sequence changes) that promoted homogeneity in the melting behavior of the amplicons. To validate conditions of analysis, we used more than 307 controls carrying 242 mutations and polymorphisms. This enabled us to determine the optimal conditions for processing the results, in particular the sensitivity because some mutations proved particularly difficult to visualize. This is particularly evident for the 1078delT mutation in exon 7, which is common in Brittany. Analysis of exon 7 does not appear challenging, and the addition of a GC clamp secures one single melting domain, but the parameter of analysis [ie, intervals of temperature for the upper (100%) and the lower (0% baseline) fluorescence lines, and sensitivity level] defined by using the most common mutations within this exon, such as R347P, do not allow visualization of the 1078delT mutation. In the absence of positive control for this mutation, exon 7 was not adequately analyzed. This case highlights the necessity of validating the analytical parameters with a large number of controls, especially when there is multiple melting domains as it is the case in most exons of genes involved in genetic diseases, particularly the *CFTR*. If the number of controls available is insufficient, the sensibility of the technique may be difficult to evaluate.

As in the study of Montgomery and colleagues, we deliberately chose to exclude 2% of the coding sequence of the gene corresponding to regions containing highly polymorphic regions such as the GATT repeat in intron 6a, IVS8 T and TG variants, as well as the M470V polymorphism.³⁰ However, in other exons, such as exons 1,

14a, and 24 there is also a high frequency of positive samples, because of the presence of polymorphisms 125G>C, T854T, and Q1463Q. In these particular cases, it is possible to combine mutation scanning and genotyping with the use of specific probes as has been done for factor V Leiden SNP and several mutations in exons 10 and 11 of the *CFTR* gene.^{10,33,34}

Although it was not the objective of this study, we also looked at the ability of the HRM technique to differentiate the melting curves of a wild-type compared to a homozygous mutated sample. As expected in ~40% cases (including the F508del) the shape of the melting curve of the two samples was not distinguishable, but this method is based on the discrimination between the heterozygous product and the wild type and not between the homozygous mutated and the wild type. The detection rates of homozygous mutations vary between 30% and 80% according to the genes and the nucleotide changes. Use of a strategy of systematic mixture of potentially homozygous samples with wild-type samples is widely documented in the literature.^{19,23,30,35} We have showed that this can be achieved before the amplification, or after a first melting. However, the ability to detect homozygotes seems to depend on the platform used.²¹ We worked on LightScanner (Idaho Technology Inc.) and have obtained, for 10 homozygous variants tested, a detection rate of 60%.

Effect of DNA quality was investigated, using old samples stored since the establishment of systematic neonatal screening in France. These carried frequent muta-

Table 3. Results of CFTR Analysis by HRM on 136 Samples of Patients with Idiopathic Chronic Pancreatitis (ICP)

Exon	Number of positive samples	Mutations identified	Variants identified	New positive controls
1	14		14 125G>C	
2	1	1 R31C		
3	9	1 G85E 1 R74W	7 R75Q	
4	4	1 R117G 1 R117H 1 A120T	1 I148T	R117G
5	1	1 L188P		L188P
6a	5	1 V201M	1 A221A 3 875+40 A>G	A221A
6b	27	1 M284T	26 1001+11C>T	M284T
7	1	1 L320V		L320V
8	0	0		
9	1	1 D443Y		
10	16	8 F508del	8 E528E	
11	1	1 G542X		
12	6	4 G576A 1 L568F	1 Y577Y	L568F
13	7	1 S737F	4 R668C 1 V754M 1 L644L	S737F L644L
14a	53		52 T854T 1 T854T+I853I	T854T+I853I
14b	0	0		
15	3	1 L967S 1 T908S 1 S945L		T908S
16	0	0		
17a	10	7 L997F 1 3271 + 3 A>G 1 Y1014C	1 3271+18C>T	3271 + 3A>G
17b	3	1 H1054D+G1069R	1 L1096L 1 3272-33A>G	L1096L H1054D+G1069R 3272-33A>G
18	2	1 D1152H 1 E1124del		E1124del
19	5		5 S1235R poly	
20	7	1 W1282X 1 D1270N	5 P1290P	
21	2	1 N1303K	1 T1299T	
22	0	0		
23	1	0	4374+13 A>G	
24	43		40 Q1463Q 2 Y1424Y 1 Q1463Q+Y1024Y	

tions, as well as negative or positive samples extracted from Guthrie cards and did not yielded satisfactory results. However, a recent study shows the feasibility of the technique for the same type of samples on the *phenylalanine hydroxylase* gene involved in phenylketonuria.²³ However, the authors had been working on a single-sample type extracted with an optimized technique.³⁶

The series of ICP patients were studied using one to six negative controls. Because the analysis is based on the comparison with the mean wild-type curve, one negative control is inadequate but two positive and two negative controls seem sufficient. This makes it possible to analyze the whole coding sequence of the *CFTR* gene for one patient in two plates, each containing 16 amplicons (32 in total). For each sample tested, six tubes are amplified (one NTC, two negative controls, two positive controls and the DNA of the patient). Analyzing the results is simplified by the use of specific subsets and analysis parameters for each

exon. Even using one thermocycler, analyzing the entire gene takes less than 1 day, including DNA extraction (1 hour), PCR twice (5 hours), two acquisitions, and 1 hour of interpretation. This is particularly important in cases of urgent results needed. For example, when an echogenic bowel is discovered during the following of a pregnancy, in our experience the diagnosis of CF is made in 10% of cases and the molecular diagnosis of CF has to be confirmed or refuted in a urgent manner.³⁷

We have been able to show in this study, the feasibility of analyzing 98% of the coding sequence of the *CFTR* by HRM. Thanks to the addition of a GC clamp, only exons 13 and 17b needed to be split into five and two fragments, respectively, contrary to the study of Montgomery and colleagues who chose to analyze exons 4, 7, 13, 15, 17b, 19, and 24 into multiple amplicons.³⁰

We showed here, that the sensitivity of the technique is melting domain-dependent and the analysis of each melt-

ing domain of a gene brings an excellent sensitivity for heterozygote detection that is very close to 100%. Analytical parameters were defined thanks to the study of more than 300 positive controls, which represents more than five times the number of mutations detected in the study of Montgomery and colleagues,³⁰ and the method is now suitable for reference diagnostic laboratories. HRM is a robust and sensitive single-tube technique to scan for mutations in a gene, which promises to become the gold standard, particularly for highly mutated genes such as *CFTR*.

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