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A 96-well formatted method for exon and exon/intron boundary full sequencing of the CFTR gene

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Abstract

Full genotypic characterization of subjects affected by cystic fibrosis (CF) is essential for the definition of the genotype–phenotype correlation as well as for the enhancement of the diagnostic and prognostic value of the genetic investigation. High-sensitivity diagnostic methods, capable of full scanning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, are needed to enhance the significance of these genetic assays. A method for extensive sequencing of the CFTR gene was optimized. This method was applied to subjects clinically positive for CF and to controls from the general population of central Italy as well as to a single subject heterozygous for a mild mutation and with an uncertain diagnosis. Some points that are crucial for the optimization of the method emerged: a 96-well format, primer project and purification, and amplicon purification. The optimized method displayed a high degree of diagnostic sensitivity; we identified a subset of 13 CFTR mutations that greatly enhanced the diagnostic sensitivity of common methods of mutational analysis. A novel G1244R disease causing mutation, leading to a CF phenotype with pancreatic sufficiency but early onset of pulmonary involvement, was detected in the subject with an uncertain diagnosis. Some discrepancies between our results and previously published CFTR sequence were found.

Keywords: CFTR; Cystic fibrosis; Extended sequencing; High-sensitivity mutational analysis

Clinical manifestations of cystic fibrosis (CF)[1] are highly variable. This is due to both intra- and extragenic causes. Intragenic variability is due to the large number of mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1] and to the even larger number of their combinations. Extragenic variability has been ascribed to the existence of modifier genes that modulate the original effect of CFTR mutations [2–6]. It is above all owing to these independent sources of variability that the genotype–phenotype correlation in CF is still poorly understood [7–11]. From the intragenic point of view, one obstacle to the understanding of the phenotypic manifestations of CFTR mutations is the often incomplete genetic characterization of subjects affected by polysymptomatic, oligosymptomatic, or monosymptomatic forms of CF. This is due mainly to technical limitations, particularly the low degree of diagnostic sensitivity of conventional methods for mutational analysis that often search for only a small number of CFTR mutations. These genetic tests are therefore of limited prognostic and diagnostic usefulness, especially in subjects with borderline clinical and/or biochemical values, where very often the only way to make a definitive diagnosis is the demonstration of CFTR mutations on both alleles. High-sensitivity methods,

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[1] *Abbreviations used:* CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DGGE, denaturing gradient gel electrophoresis; SSCP, single-strand conformation polymorphism; DHPLC, denaturing high-performance liquid chromatography; IRT, immunoreactive trypsinogen; EDTA, ethylenediaminetetraacetic acid; TEAA, triethylammonium acetate.

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capable of fully scanning the gene, are needed to resolve uncertain diagnoses, to clarify the genotype–phenotype correlation, and to enhance the prognostic value of the genetic test. During past years, several methods (for a review, see Ref. [12]) capable of enhancing conventional methods of direct search for specific CFTR mutations have been developed and/or optimized specifically for CFTR scanning: denaturing gradient gel electrophoresis (DGGE) [13,14], single-strand conformation polymorphism (SSCP) and heteroduplex analysis [15–17], denaturing high-performance liquid chromatography (DHPLC) [18–20]. This enhanced sensitivity is not, however, great enough to allow a "full" genetic characterization. To address this issue, we optimized a 96-well formatted method for the sequencing of the 5′ flanking region, all of the exons, and the adjacent intronic regions of the CFTR gene. This method was applied, after being validated on 26 subjects from the general population, to 61 CF-affected persons and to one subject who had escaped neonatal screening for CF because the first immunoreactive trypsinogen (IRT) dosage had been negative but who showed CF-like symptoms of uncertain significance and carried a known mild CF mutation.

Materials and methods

Specimen collection, biochemical and clinical characterization, DNA extraction

A total of 26 blood samples from subjects randomly chosen from the general population were analyzed to test the CFTR extended sequencing procedure. None of these subjects showed any clinical manifestations of CF, nor were any positive for CFTR mutations when analyzed by means of the PCR/OLA/SCS method (Celera Diagnostics) [21], which searches for the most common worldwide 31 CFTR mutations (G85E, R117H, Y122X, 621+1G->T, 711+1G->T, 1078delT, R347P, R347H, R334W, A455E, ΔF508, ΔI507, Q493X, V520F, 1717-1G->A, G542X, G551D, R553X, R560T, S549R(T->G), S549N, 1898+1G->A, 2183AA->G, 2789+5G->A, R1162X, 3659delC, 3849+10kbC->T, 3849+4A->G, W1282X, 3905insT, N1303K), including the 12 most common in Italy [1,22]. A total of 61 subjects affected by CF were also studied. Of these, 49 had been previously found, by the PCR/OLA/SCS assay, to carry one CFTR mutation, with the other remaining unknown (mutation/unknown genotype); the remaining 12 subjects were selected because no CFTR mutations were detected by the PCR/OLA/SCS assay (unknown/unknown genotype). In addition, 1 subject with a mutation/unknown genotype, namely a mild 3849+10kbC->T mutation in heterozygosity, was selected despite a negative result at the first neonatal IRT assessment, as a case of "uncertain diagnosis" owing to the presence of some clinically significant CF symptoms and laboratory results, including pulmonary microbial colonization, sweat chloride test [23], stool chymotrypsin (Chymo, Roche, Mannheim, Germany) [24] and elastase-1 (E1 stool test, ScheBo-Tech, Giessen, Germany) [25] determinations, steatorrhea [26]. Therefore, we analyzed, by the sequencing procedure, 52 supposedly normal alleles from healthy subjects and 74 potentially CF alleles belonging to affected subjects that, however, lacked any of the 31 most common CFTR mutations. To verify the absence of the novel G1244R mutation in the general population, the exon 20 of a further 90 subjects from the general population was analyzed. In all of our analyses, the DNA of all the samples was extracted by means of the Qiagen DNA blood midi kit using 2 ml of frozen blood in ethylenediaminetetraacetic acid (EDTA); blank samples (water instead of blood) were extracted together with the samples. The blank samples were subjected to all of the other steps of this procedure (from PCR amplification to sequencing) to rule out cross-contamination.

Extended sequencing: PCR amplification and check and purification of amplicons

Each CFTR zone was amplified and sequenced by using external and internal primers, respectively. This approach ensures a greater analytical specificity, without affecting efficiency, if compared with the use of universal sequencing tags on the PCR primers, preventing the sequencing of nonspecific PCR products that may be present at undetectable levels. Different pairs of external primers (Table 1) originally were designed for full amplification of the 5′ flanking region (2 amplicons needed) and of all the exons and adjacent intronic regions (2 amplicons needed for exon 13 and 1 amplicon needed for every other exon), amounting to 30 amplicons for each subject. Some of these primers partially overlapped with those of Zielenski and coworkers [27]. The forward primer for exon 9 was the one used by Chu and coworkers [28]. To avoid the loss of amplicons due to interfering sequence variations, the presence of polymorphisms possibly overlapping the primer sequence was checked by means of the database of the Consortium for CF Genetic Analysis [1]. Of the 58 external primers reported, only 5 were found to have known polymorphisms overlapping their sequence: the forward external primer for exon 10 (2 polymorphisms), the forward external primers for exons 19 and 23 (1 polymorphism each), and the backward external primers for 5′ flanking 5′ side and for exon 16 (1 polymorphism each). A minimal frequency of false results, possibly due to lack of amplification, is expected.

The 30 pairs of external PCR primers were divided into four groups. Each group was designed to contain primers with an annealing temperature of 54 °C (two groups), 58 °C, or 62 °C. An experimental plate in a 96-well format (Fig. 1; see also Excel protocol template in Supplemental data), arranging 8 exons × 12 samples to amplify simultaneously, can be prepared for each group of primers. All of the manipulations can be performed using multichannel pipettors (or automated workstations). All of the reagents, initially assembled into a general master mix without primers, were partitioned in 8 aliquots, assembled in an 8-well

Table 1
External primers and annealing temperatures used for PCR amplification

Exon	PCR external primer sequences for group I of exons		bp
	Forward	Backward	
(A) 54 °C T_a			
4	5′-CTCCCACTGTTGCTATAACAAATCCC-3′	5′-AGCATTTATCCCTTACTTGTACCAGC-3′	527
11	5′-AAATTGCATTTGAAATAATGGAGATGC-3′	5′-AAGATACGGGCACAGATTCTGAGTAACC-3′	475
14a	5′-TCATTTTTAATAAAGCTGTGTTGCTCCAG-3′	5′-AGTGGTTCTACTTGTTGATTTTTCAGAAGC-3′	595
17a	5′-AAAAAGTTTGAGGTGTTTAAAGTATGC-3′	5′-CACCAACTGTGGTAAGATTCTATATACC-3′	527
17b	5′-TCAAAGAATGGCACCAGTGTG-3′	5′-AATTGCTACTCGACAATCTGTGTGCATC-3′	700
18	5′-CTGGTTGAATACTTACTATATGCAGAGC-3′	5′-AAGGAAACAGGTGAAAGAATGC-3′	536
19	5′-CCGACAAATAACCAAGTGACAAATAGC-3′	5′-GTTACAAATAGATTCTGCTAACACATTGC-3′	468
20	5′-GGTCAGGATTGAAAGTGTGCAACAAGG-3′	5′-TCCCAAACTTTTAGAGACATCTTTTCTGCC-3′	502
	PCR external primers sequences for group II of exons		
	Forward	Backward	
(B) 54 °C T_a			
8	5′-TCAAATATGATGAATCCTAGTGCTTGGC-3′	5′-CTTGGTCTCCCGAAAGTGCTGG-3′	396
9	5′-TTGATAATGGGCAAATATCTTAG-3′	5′-CCTTCCAGCACTACAAACTA-3′	483
10	5′-CACTTCTGCTTAGGATGATAATTGGAGG-3′	5′-TTCATGTGTTTGCAAGCTTCTTAAAGC-3′	467
13[a]	5′-GAATTCACAAGGTACCAATTTAATTACTAC-3′	5′-AGTTATCACTGGCTTAGTAGAGGACC-3′	1162
13[a]	5′-GAATTCACAAGGTACCAATTTAATTACTAC-3′	5′-AGTTATCACTGGCTTAGTAGAGGACC-3′	1162
14b	5′-CAAATGGTGTGATGTGAATTTAGATGTGGG-3′	5′-AATGCTTGGGAGAAATGAAACAAAGTGG-3′	243
16	5′-TCAGAGAAATTGGTCGTTACTTGAATC-3′	5′-ATTGCCTCAGGTTTGGGCCAG-3′	558
24	5′-TGCAAGGCTCTGGACATTGC-3′	5′-CCAGGAAGCCATTTATCAAGACC-3′	522
	PCR external primers sequences for group III of exons		
	Forward	Backward	
(C) 58 °C T_a			
3	5′-GAGTGTTTGGTGTTGTATGGTCTCC-3′	5′-TTACTTATCCTTACTAGAGTTTTAGGTGG-3′	431
5	5′-TGACTGTTGAAAGAAACATTTATGAACC-3′	5′-TCCAGAATAGGGAAGCTAGAGCTGAGC-3′	506
6a	5′-TACCTAGATTTTAGTGTGCTCAGAACCACG-3′	5′-AAGATCGTGCCACTGCACTCCAGC-3′	491
6b	5′-GTACAGCGTCTGGCACATAGGAGG-3′	5′-CAGCCCATGAAAGTGAATTTGTGC-3′	493
15	5′-TAATAGTATGATTTTGAGGTTAAGGGTGC-3′	5′-CACATGCCTCTGTGCAAAAGCC-3′	506
21	5′-ATTGGATTAGAAAAATGTTCACAAGG-3′	5′-GAATGATCTTAGCCATATCAACC-3′	571
22	5′-TTTGCTCAATCAATTCAAATGGTGGC-3′	5′-CCACTGGGCAATTATTTCATATCTTGGG-3′	466
23	5′-GGTTGAAAAGCTGATTGTGCGTAACG-3′	5′-AGCTATTTTGAGTAAAGCTGGATGGC-3′	420
	PCR external primers sequences for group IV of exons		
	Forward	Backward	
(D) 62 °C T_a			
5′ flanking 5′ side	5′-GCCCCTCAGAGAGTTGAAGATGGCG-3′	5′-CTCTCTTTAGGTCCAGTTGGCAACG-3′	716
5′ flanking 3′ side	5′-TAACAGGAACCCGACTAGGATCATCG-3′	5′-CTGTGATGTCATTTGCTTCCAATTCC-3′	531
1	5′-TAACAGGAACCCGACTAGGATCATCG-3′	5′-CCCCAAACCCAACCCATACACACGCCC-3′	771
2	5′-CAGGTGTAGCCTGTAAGAGATGAAGCC-3′	5′-GCTGGTATCACAAACTCCTGGTCTCAAGC-3′	481
7	5′-ATAACATGCCCAAGGTCACACAGG-3′	5′-GGTGAACATTCCTAGTATTAGCTGGC-3′	564
12	5′-TGCATGTAGTGAACTGTTTAAGGC-3′	5′-ATGGAAGTAATCTTGAATCCTGGCC-3′	364

Note. These primers originally were designed for this work. Some of them were found to partially overlap with those of Zielenski and coworkers [26]. The forward primer of exon 9 is that of Chu and coworkers [27]. The bp column indicates the size of amplicons.

[a] Exon 13 was amplified twice, by the same primer pair, so as to maintain the 96-well format in the subsequent sequencing step.

strip, and the appropriate pair of primers was added to each aliquot so as to produce 8 primer-containing master mixes to dispense. The DNA samples were arranged in a 12-well strip (11 experimental samples +1 blank) and dispensed. This arrangement crosses the primer pairs with samples so that each well in the 96-well plate contains a unique combination of samples and primer pairs to amplify a unique CFTR zone for that sample. The final total volume in each well is 15 μl. Each DNA sample originally was diluted to obtain a final quantity of 150 ng of DNA for 15 μl of reaction volume. The primer concentration initially was adjusted to obtain a total amount of 6 pmol in the 15 μl of reaction volume. The general master mix initially was diluted to obtain the following final concentrations in 15 μl of reaction: 0.5 U of the YieldAce Hotstart DNA polymerase (Stratagene), 1× of provided reaction buffer (with Mg^{2+}), and 175 μM of each dNTP. The use of antibody-based Hotstart DNA polymerase allows a room temperature PCR setup. The four exon groups were amplified, one at a time, on the 96-well supporting PCR cycler PTC-100 (MJ Research). The annealing temperatures were 54 °C for two primer groups and 58 and 62 °C for the other two primer groups

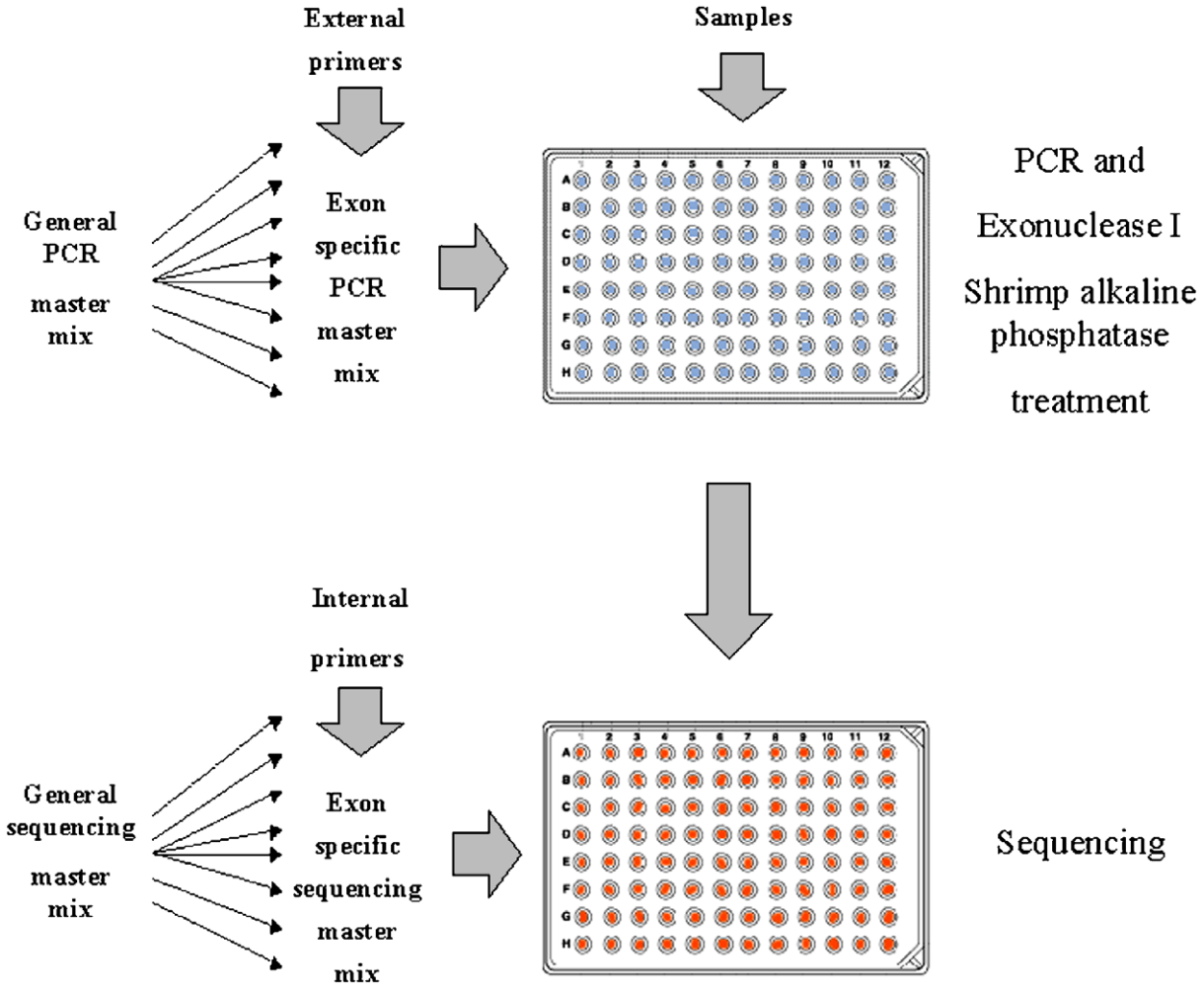

Fig. 1. Schematic representation of 96-well formatted experimental design. (See also Excel protocol template in Supplemental data).

(Table 1). This procedure yielded the full amplification of CFTR for 11 samples plus the blank by means of four successive amplifications. A similar PCR cycle was used for all of the exon groups: a denaturation prestep at 92 °C for 2 min; followed by 35 cycles at 94 °C for 45 s; at 54, 58, or 62 °C for 1.5 min; and at 72 °C for 2.5 min; followed by a final extension at 72 °C for 7 min.

A preliminary assessment of the amplification efficiency, of possible nonspecific amplification, and of possible contamination was made by 1% agarose gel electrophoresis and ethidium bromide staining of 3 µl of amplicons, performed using standard procedures [29]. This step was also optimized in a 96-well format by using 14-tooth combs at a multichannel pitch to create four rows of wells in one gel. The 1st and 14th teeth in each row were used for a molecular weight marker, whereas the 12 central teeth were used for 11 samples plus the blank (loaded by 12-channel pipettor). All 96 samples were run by an initial loading of the first 48 samples, followed by a 30-min run at 5 V/cm, and by a further loading of the second 48 samples, followed by a 1-h run. Thus, the simultaneous visualization of the amplicons (size range shown in Table 1) of the whole 96-multiwell plate was achieved by using a single agarose gel.

It is imperative, before performing the subsequent cycle sequencing reactions on amplicons, that both residual primers and unincorporated dNTPs be eliminated. This step is necessary even if the PCR products are very clean. Two different kinds of amplicon treatment were tested alternatively: chromatographic purification and enzymatic treatment. For chromatographic purification, the QIAquick 96 PCR Purification Kit (Qiagen) and the QIAvac 96 manifold (Qiagen) were used according to the manufacturer's instructions. Enzymatic treatment was instead performed by adding, to each amplicon, 12 U of Exonuclease I (MBI Fermentas) and 1.2 U of shrimp alkaline phosphatase (Amersham Pharmacia Biotech) in the presence of 1× Exonuclease buffer provided by the manufacturer, also resulting in the original volume of 15 µl, which had been subtracted in the electrophoretic step, being restored in each well. This treatment was performed for 45 min at 37 °C, followed by heat inactivation at 80 °C for 15 min. Both procedures can be performed easily in a 96-well format. After the purification step, the amplicons

were assessed by agarose gel electrophoresis as described above; no significant reduction in signal intensity (amplicon quantity) was detected after either treatment. Both methods appeared to work well and are, in our experience, suitable for the method proposed in this article.

Extended sequencing: Cycle sequencing reaction and purification, capillary electrophoresis, sequence analysis

The internal primers (Table 2) used for the sequencing of 5′ flanking (both 5′ and 3′ sides) and exons 1, 2, 3, 6a, 6b, and 21 (both forward and backward) originally were designed for this work. All of the other internal primers were designed by Fanen and coworkers [13] for DGGE analysis, although they were synthesized for this work without the GC tail. These internal primers allow the detection of the first and last known mutations potentially present at each exon/intron boundary, spanning 972 of a total of 984 known CFTR mutations [1]. The 12 mutations not included in this assay are 10 large deletions and 2 full intronic mutations (although 1 is included in the PCR/OLA/SCS assay). Our assay also detects a large part of the 222 known polymorphisms of the CFTR gene [1]. The sequencing of purified amplicons was performed using

Table 2
Internal primers used for cycle sequencing

Exon	Sequencing internal primers sequences for group I of exons	
	Forward	Backward
A		
4	5′-TGTGTTGAAATTCTCAGGGT-3′	5′-CAGAATATATGTGCCATGGG-3′
11	5′-CAGATTGAGCATACTAAAAGTG-3′	5′-CATTTACAGCAAATGCTTGCTAG-3′
14a	5′-GGTGGCATGAAACTGTACTG-3′	5′-TGTATACATCCCCAAACTATCT-3′
17a	5′-TGCAATGTGAAAATGTTTAC-3′	5′-CTCTTATAGCTTTTTTACAA-3′
17b	5′-TTTGTGTTTATGTTATTTGC-3′	5′-TGCAGCATTTTATTCATTGA-3′
18	5′-TAGGAGAAGTGTGAATAAAG-3′	5′-ATACTTTGTTACTTGTCTGA-3′
19	5′-GTGAAATTGTCTGCCATTCT-3′	5′-AGGCTACTGGGATTCACTTA-3′
20	5′-TATGTCACAGAAGTGATCCC-3′	5′-TGAGTACAAGTATCAAATAGC-3′
	Sequencing internal primers sequences for group II of exons	
	Forward	Backward
B		
8	5′-TAAAGTAGATGTAATAATGC-3′	5′-ATTTTATTCGCCATTAGGAT-3′
9	5′-TGAAAATATCTGACAAACTC-3′	5′-CCTTCCAGCACTACAAACTA-3′
10	5′-TCCTGAGCGTGATTTGATAA-3′	5′-ATTTGGGTAGTGTGAAGGG-3′
13 5′ side	5′-CAAAATGCTAAAATACGAGACA-3′	5′-TCCCTGCTCAGAATCTGGTA-3′
13 3′ side	5′-CCCTTACAAATGAATGGCAT-3′	5′-TACATATTGCATTCTACTCA-3′
14b	5′-AATAGGTGAAGATGTTAGAA-3′	5′-ATAAAACACAATCTACACAA-3′
16	5′-TCTGAATGCGTCTACTGTGA-3′	5′-GCAATAGACAGGACTTCAAC-3′
24	5′-TTTCTGTCCCTGCTCTGGTC-3′	5′-TCCCACGAGCTCCAATTCCA-3′
	Sequencing internal primers sequences for group III of exons	
	Forward	Backward
C		
3	5′-TCTATAATACTTGGGTTAATCTCCTTGG-3′	5′-TAGTGTTTGGAGTTGGATTCATCC-3′
5	5′-TATTTGTATTTTGTTTGTTGA-3′	5′-CTTTCCAGTTGTATAATTTA-3′
6a	5′-TCTAGGGGTGGAAGATACAATGACACC-3′	5′-AGAGCAGTCCTGGTTTTACTAAAGTGGG-3′
6b	5′-TGATAATAAAATAATGCCCATCTGTTG-3′	5′-TCAAATATGAGGTGGAAGTCTACCATG-3′
15	5′-TCAGTAAGTAACTTTGGCTGC-3′	5′-CCTATTGATGGTGGATCAGC-3′
21	5′-TTTGAGAGAACTTGATGGTAAGTACATGGG-3′	5′-AATCATTTCAGTTAGGGGTAGGTCCAGTC-3′
22	5′-TTTTAGAATGTCAACTGCTT-3′	5′-ATGATTCTGTTCCCACTGTG-3′
23	5′-CTGTTCTGTGATATTATGTG-3′	5′-GTTATCAAGAATTACAAGGG-3′
	Sequencing internal primers sequences for group IV of exons	
	Forward	Backward
D		
5′ flanking 5′ side	5′-GCCCCTCAGAGAGTTGAAGATGGCG-3′	5′-CTCTCTTTAGGTCCAGTTGGCAACG-3′
5′ flanking 3′ side	5′-TAACAGGAACCCGACTAGGATCATCG-3′	5′-CTGTGATGTCATTTGCTTCCAATTCC-3′
1	5′-AGGGAGGCTGGGAGTCAGAATCG-3′	5′-CCCCAAACCCAACCCATACACACGCCC-3′
2	5′-ATTCCAAATCTGTATGGAGACCAAATCAAG-3′	5′-CAAATAGTTGGGATTACAGGCATTAGCCAC-3′
7	5′-CATCCTGAATTTTATTGTTA-3′	5′-ATCATAGTATATAATGCAGC-3′
12	5′-ATGACCAGGAAATAGAGAGG-3′	5′-GCTACATTCTGCCATACCAA-3′

Note. The primers of the 5′ flanking (both 5′ and 3′ sides) and of exons 1, 2, 3, 6a, 6b, and 21 (forward and backward) originally were designed for this work. The other primers are those used by Fanen and coworkers [13] but deprived of the GC tail.

the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly (Fig. 1; see also Excel protocol template in Supplemental data), for each exon group, a general cycle sequencing master mix (protocol with 0.5× ready reaction premix and BigDye Sequencing Buffer according to manufacturer's instructions) was partitioned in 8 aliquots and the specific sequencing primers were added (to a final quantity of 5 pmol/reaction), thereby obtaining 8 different exon-specific mixtures. Aliquots of 6 μl were dispensed into a new 96-well sequencing plate, followed by the addition of 4-μl aliquots of each amplicon. The final volume of each cycle sequencing reaction was 10 μl. Each well contains a unique combination of amplicon and specific sequencing primer. Starting from each amplification plate, two sequencing plates (one for the sequencing of the forward strand and the other for the sequencing of the backward strand) can be assembled easily using multichannel pipettors (or automated workstations). All of the internal sequencing primers can be used at the same annealing temperature. A general cycle was used for all of the cycle sequencing reactions: a denaturation prestep at 96 °C for 1 min, followed by 30 cycles at 96 °C for 10 s, at 50 °C for 5 s, and at 60 °C for 4 min.

The removal of unincorporated fluorescent ddNTPs was performed, in a 96-well format, by using the DyeEx 96 kit (Qiagen) according to the manufacturer's instructions. Briefly, all 10 μl of the cycle sequencing reaction was added to the DyeEx plates, and a pair of DyeEx plates (forward and backward sequencing) was centrifuged on a T517 swing-out rotor in a PK121R ALC centrifuge at 1000*g*.Denaturation was performed by adding 30 μl formamide to each well and treating at 92 °C for 2 min.

The electropherograms were developed by the four-capillary ABI PRISM 3100 *Avant* genetic analyzer (Applied Biosystems), using a 36-cm array and POP6 polymer, under standard prerun and run conditions. Raw data were collected by means of the Collection software, preliminary analysis was performed by the Sequencing software, and detailed subsequent analysis, including an assessment of the quality value for each base, was performed by means of the SeqScape software (all from Applied Biosystems).

DHPLC assay design

With the aim of optimizing a rapid method for the search of the novel G1244R mutation on a large number of samples, a DHPLC assay was designed and performed by a WAVE DNA Fragment Analysis System on a DNASep column (Transgenomic). The amplicons of CFTR exon 20 were obtained by means of the pairs of internal primers shown in Table 2, under the PCR conditions described above, using 54 °C as the annealing temperature. Amplicons were denatured for 5 min at 95 °C. Heteroduplex formation was obtained by allowing gradual reannealing through exposure to room temperature over a 15-min period. Chromatographic runs (6 min) were performed at a flow rate of 0.9 ml/min using a linear acetonitrile gradient consisting of buffer A (0.1 M triethylammonium acetate [TEAA])/buffer B (0.1 M TEAA and 25% acetonitrile) with a start gradient of 53.7% and a gradient increase of 2%/min of buffer B, using a temperature of 57.3 °C for maximal heteroduplex separation. The analysis was performed by the Navigator 1.5.3 software (Transgenomic).

Results

Optimization of the extended sequencing procedure and analysis of subjects from the general population

Some points that are crucial for the successful performance of extensive mutational analysis by sequencing emerged from the results. First, the use of the 96-well format was found to be a great advantage when treating a large amount of samples in all of the protocol steps: PCR amplification, amplicon purification, cycle sequencing, sequence purification, and instrumental analysis. This protocol can be applied easily by manual pipetting using multichannel pipettors or can even be transferred to automated workstations with no substantial modifications. Second, the simultaneous amplification of different CFTR segments, achieved by groups of primers sharing the same annealing temperature and the same PCR cycle, greatly reduced the time needed for analysis. Third, good purification of the PCR products was achieved by using either the chromatographic method or the enzymatic method. One of these methods usually was sufficient to reduce background due to residual PCR primers to an undetectable level. In a very small number of cases (after the chromatographic step or even in protocols without chromatographic purification), an additional enzymatic treatment was required to eliminate background completely. Fourth, in the cycle sequencing step, either HPLC-purified or nonpurified primers could be used, although in some cases the latter induced a somewhat higher $n - 1$ background. The combined use of the PCR purification enzymatic step and HPLC-purified primers for cycle sequencing proved to be a good combination, with excellent background elimination, maximization of the signal/noise ratio, and manageability. Fifth, the use of dedicated high-level analysis software, such as SeqScape, also allowed partial automation of the analysis step.

In our experience, a complete forward and backward scanning of 11 samples plus the blank can be achieved, by a well-trained operator, using the 4-capillary ABI PRISM *Avant* genetic analyzer, in approximately 2 weeks with the following approximate time frame: 3 days for PCR, electrophoretic control, and amplicon purification; 3 days for sequencing and further purification; and 1 week for instrumental time (the treatment of the 4 plates, both forward and backward, requires a running time of ~160 h) and for sequence analysis (which can be performed on completed runs while the remainder are still running). Instrumental time can be reduced drastically to 40 h by

using a 16-capillary analyzer and to less than 8 h by using a 96-capillary analyzer. The use of dedicated software for semiautomated sequencing analysis, such as SeqScape, greatly improves the analysis phase. Indeed, extended scanning is required in only a small number of samples because some samples reach complete genetic characterization during analysis of the initial plates. From this point of view, a sequential approach, as opposed to contemporary treatment using 4 plates, should be preferred and those exons with the best sensitivity should be chosen (for alleles that are unknown after the 31-mutation assay) (see below).

No CFTR mutations were found in subjects from the general population. Some discrepancies emerged between our sequence and previously published CFTR sequence (Table 3).

Novel G1244R mutation and biochemical and clinical characterization of a subject with uncertain diagnosis

The application of this protocol to the subject with an uncertain diagnosis allowed us to detect the novel CFTR mutation G1244R (3862 G->A, glycine to arginine) in exon 20 (Fig. 2A) [1] in the critical NBF2 domain. The analysis of allelic segregation in the parents assigned the 3849+10kbC->T (carried by the mother) and G1244R (carried by the father) mutations to different alleles. No other mutations were found in this subject. The novel G1244R mutation was not found in the other 73 unknown CF alleles analyzed, nor was it found in the 232 alleles from the general population. This mutation can be detected easily even by DHPLC because the chromatogram was found to be characteristically modified (Fig. 2B).

The biochemical and clinical characteristics of the 3849+10KbC->T/G1244R genotype are as follows. The family ethnic origin was central Italy. The subject came to the CF center at 14 months of age, showing mild lung involvement with past pneumonia at 3 months, persistent catarrhal cough, repeated wheezing, and slight pulmonary emphysema. The sweat test, repeated three times in different years, yielded a constantly borderline value of Cl^- concentration: average 54.3 mEq/L ($\pm$ 1.2). This subject was pancreas sufficient, as ascertained from the nonpathological levels of stool elastase-1 (>500 μg/g in two independent dosages performed in 2 different years) and chymotrypsin (>20 U/g in three independent dosages performed in 3 different years) as well as from the absence of steatorrhea. However, weight was moderately reduced (from a maximum of the 15th centile to a minimum of the 6th centile), although height was normal (never below the 23rd centile). Neither meconium ileus nor liver involvement was found, but nasal polyposis was observed. Occasional lung colonization by nonmucoid *Pseudomonas aeruginosa* and persistent colonization by *Staphylococcus aureus* were also found. This subject escaped an IRT/DNA/IRT protocol of neonatal screening for CF [30] because the first IRT dosage was below the cutoff. From a clinical point of view, the borderline sweat test value and the persistent colonization by *S. aureus* strengthened the likelihood of disease. After enrollment at the CF center and subsequent treatments, no other episodes of pneumonia occurred (current age 5 years).

Diagnostic sensitivity increment by extended mutational analysis of a specific subset of CFTR exons

The PCR/OLA/SCS mutational panel, including the 31 most common CFTR mutations (Celera Diagnostics), is 77.6% sensitive in central Italy (our unpublished results). In this work, we found a limited subset of 13 mutations (not included in the PCR/OLA/SCS assay) in 7 CFTR exons, significantly improving the sensitivity of standard assays: D110H, R117C, and H139R (exon 4); R334L, T338I, and A349V (exon 7); S549R(A->C) (exon 11); Y849X (exon 14a); L997F (exon 17a); L1065P, R1066C, and L1077P (exon 17b); and G1244E (exon 20). The increase in sensitivity that can be achieved by analyzing these mutations is 10.0%, as calculated from our data. The possibility of analyzing a limited number of exons, with the greatest experimental calculated diagnostic sensitivity and formatted into a unique 96-well plate assay, can further reduce the need to perform the entire protocol.

Discussion

Although PCR amplification and sequencing previously have been used [27,31] to initially characterize the CFTR gene, the main obstacles to the widespread use of these techniques for mutational analysis of large genes, such as CFTR, are the complexity and costs of the assay when handling a large number of samples. This work has highlighted some steps that appear to be crucial for the successful per-

Table 3
Discrepancies with CFTR published sequence

5′ flanking	−266 A (instead of C)	Published	GGGCGGTCAGGACACTGACCTGG
	−258 G (instead of T)	This work	GGGCGGTAAGGACACGGACCTGG
Intron 8	1342 − 195 + T	Published	TTAGTTT-AGATCAT
		This work	TTAGTTTTAGATCAT
	1342 − 159 + T	Published	ATATAAT-ATGTACT
		This work	ATATAATTATGTACT
Intron 17a	3272 − 57 − A	Published	TATTATGAAAATTAC
		This work	TATTATGAAATTAC
Intron 20	4005 + 121 − TT	Published	TGCTGTCTTTTTTTTC
		This work	TGCTGTCTTTTTTC

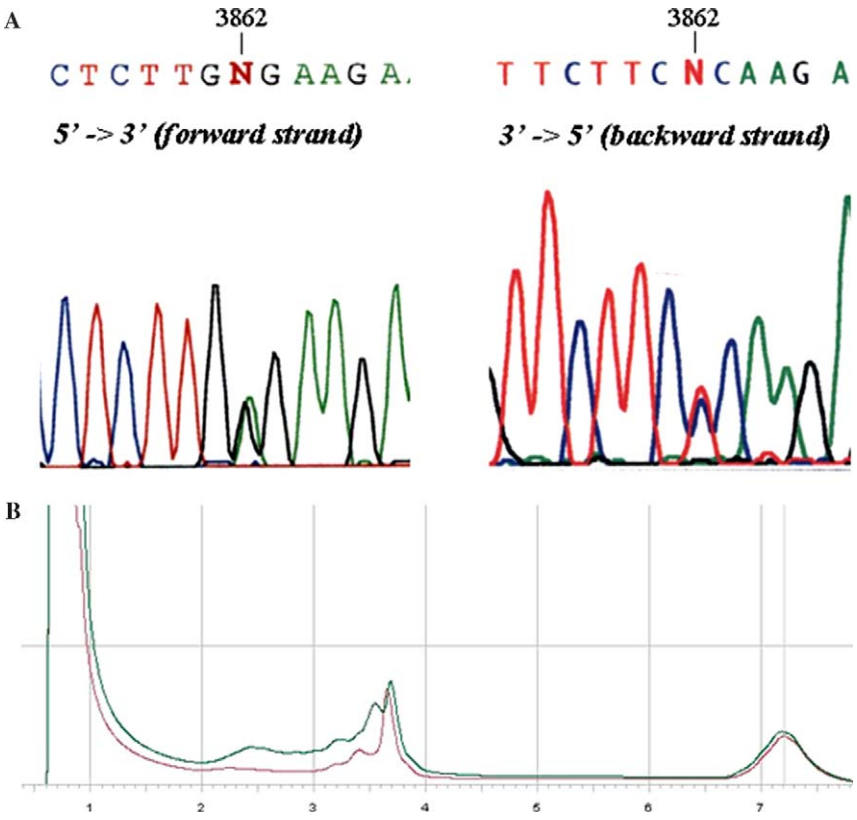

Fig. 2. The G1244R mutation by sequencing (A) and by DHPLC (B). In panel B, software comparison between heterozygous (green) and normal (red) profiles.

formance of extensive mutational analysis by sequencing: 96-well format throughout the protocol, use of HPLC-purified primers for sequencing to reduce $n-1$ background, optimization of primer annealing temperatures and of PCR cycles for simultaneous amplification, enzymatic purification of amplicons, and use of multicapillary genetic analyzers. Following these steps when performing the assay reduces analysis time and facilitates the handling of a large number of samples. To reduce the overall costs to reasonable levels even in large-scale use, the assay was also designed to reduce both the total volume of reactions and the relative amount of sequencing terminator mix.

We found five single-base discrepancies and 1 double-base discrepancy between our sequence and previously published CFTR sequence. These discrepancies may lead to a loss of amplification (false-negative PCR) if one attempts amplification using primers designed on the basis of inaccurate sequences. This problem may affect the accuracy not only in sequencing-based assays but also, more generally, in all of the PCR-based assays used in molecular genetics. These errors were found mainly in base-repeat stretches, which are those zones in which first-generation sequencing assays displayed the lowest reliability. Moreover, several individual sequence variations (polymorphisms), which may affect primer binding in PCR assay, were also found in both the general and CF populations analyzed. To limit polymorphism-based loss of amplification, CFTR zones with a minimal density of uncommon polymorphisms [1] were selected as primer target sites in the assay presented in this work.

In our opinion, the sequencing approach using new-generation sequencing assays is essential to validate published sequences on large numbers of individuals, to correct possible errors, and to find individual polymorphisms that may interfere with common techniques of mutational analysis.

Several findings suggest that the novel G1244R should be classified as a disease-causing mutation. Amino acidic substitution (glycine to arginine) changes the aminoacidic charge drastically; moreover, it arises in the critical NBF2 domain, which is crucial for ATP binding. The importance of the 1244 codon, which can be classified as a mutational hot spot, is also highlighted by the fact that this same codon can be affected by two other mutations, G1244V [32] and G1244E [33], both of which already have been shown to cause disease. The G1244R mutation was absent from the 232 alleles in the general population and in the other affected subjects studied, and it was found as the only mutation on one allele of 1 other subject studied, with no other mutation on this allele being detectable by extended sequencing. The 3849+10kbC->T mutation, which belongs to class V of CFTR mutations and was present on the other CFTR allele of the affected subject, has already been reported to occur with a variable phenotype that is generally mild [8,34–38]. In contrast, the clinical phenotype of the subject analyzed is a CF phenotype with early onset of pulmonary involvement, albeit with pancre-

atic sufficiency and a non-fully positive sweat test. Nevertheless, additional functional studies should be performed to assess the exact clinical severity of the G1244R mutation. The fact that this subject escaped neonatal screening demonstrates that both the G1244R and 3849+10kbC->T mutations yield a negative neonatal IRT dosage; with regard to the latter mutation, this finding is in contrast to some previous reports [39]. Therefore, it may be advisable to include a search for mutations with a negative or uncertain neonatal screening result in the first step of neonatal screening protocols. The frequency of the G1244R mutation must be calculated on a larger number of CF subjects to ascertain whether it is a rare mutation; however, the frequency of the G1244E mutation, one of the other mutations in the same codon, was found to be approximately 0.3%, which is relatively high.

The optimized method for the extended sequencing of the CFTR gene displayed a high degree of diagnostic sensitivity. Nevertheless, the method retains sufficient experimental plasticity to allow the treatment of a single sample, by analyzing all the exons in one step in a 96-well format, as well as of a large number of samples. Methods such as this facilitate the diagnosis of genetic diseases due to large genes with high mutational rates and resulting in a variety of clinical phenotypes that render diagnosis more difficult. CF is a clear example of this problem: the diagnosis in a large number of individuals, with atypical clinical and biochemical manifestations, remains uncertain until an extensive CFTR mutational analysis reveals both mutations.

A sequential approach, performed by selecting groups of exons with the highest sensitivity for each geographical area, can greatly reduce the need to scan the entire gene, thereby reducing both costs and time. This approach also allows the selection of a limited number of mutations (e.g., the 13 reported here for central Italy) that can then be formatted in rapid high-sensitivity tests. For example, one could choose a sequencing-based 96-well formatted exon-specific subprotocol for the search of all mutations in selected exons or, alternatively, some method of direct search for specific mutations, including the best panel selected previously by extended sequencing. In central Italy, for instance, greatly enhanced sensitivity could be achieved, if compared with that of standard panels, by an additional search for the previously mentioned 13 mutations in 7 exons, yielding an overall sensitivity for a two-step procedure (standard panel +7 exons sequencing) of approximately 88%. The application of this approach to a larger number of cases would yield even more accurate data on the extent of this increase in diagnostic sensitivity.

The CFTR gene displays several polymorphic zones in terms of both polyvariant repeated tracts, such as the $(TG)_mT_n$ in intron 8, and single-base substitutions [27]. It has already been ascertained that CFTR polyvariant tracts can be studied successfully by sequencing [40] and that such sequences may be relevant in uncertain diagnoses. However, it is difficult to solve these sequences by scanning techniques alone without subsequent sequencing. A mean of six polymorphic single-base substitutions for each subject could be calculated on the basis of the data collected in this work. As a consequence, if a scanning technique were applied, an average of six sequences would need to be performed for each subject after scanning to characterize suspect electrophoretic patterns. These considerations on polyvariant tracts and polymorphisms, combined with the known loss in sensitivity of gene-scanning procedures, suggest that a multiwell formatted sequencing approach on multicapillary genetic analyzers, without a previous scanning step, may serve as a means of achieving maximal diagnostic sensitivity using an assay while reducing both the complexity and the costs involved in such searches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.03.022.

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