Development of a method to detect three frequent mutations in the CFTR *gene using allele-specific real time PCR*

TECHNIQUE

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. A method was developed to detect three frequent CFTR mutations (F508del, 3120+1G>A and N1303K), using allele-specific real time PCR (ASPCR). Genomic DNA samples from nine patients carrying different CFTR mutations were evaluated in triplicate. The Ct variation (ΔCt) between wild type and mutant ASPCRs, and the mean value ± standard deviation (SD) were determined. The specificity of allele-specific primers was confirmed by High Resolution Melting (HRM) analysis. The results validated this test for genotype determination; the tested mutations were clearly identified in all the samples and in excellent agreement with the commercial panel StripAssay (Viennalab, Austria). The described method provides the necessary selectivity and specificity as required for clinical screening of CF population, mainly for F508del mutation due to its high prevalence among the Cuban population.

Keywords: CFTR gene mutations, allele-specific real time PCR, Ct variation, Cystic fibrosis

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RESUMEN

Desarrollo de un método para la detección de tres mutaciones frecuentes en el gen CFTR mediante PCR en tiempo real alelo-específica. La fibrosis quística (FQ) es una enfermedad autosómica recesiva provocada por mutaciones en el gen regulador de la conductancia transmembranal de la fibrosis quística (CFTR). Se desarrolló un método para detectar tres mutaciones frecuentes en el gen CFTR (F508del, 3120+1G>A and N1303K), mediante PCR en tiempo real alelo-específica (ASPCR). Se evaluaron por triplicado las muestras de ADN genómico de nueve pacientes que portaban las mutaciones CFTR mencionadas, y se determinó la variación de Ct (ΔCt) entre las reacciones de ASPCR para los genotipos mutado y salvaje, expresada como media ± la desviación estándar. La especificidad de los cebadores por los alelos se confirmó mediante análisis de Fusión de Alta Resolución (HRM). Los resultados validaron la utilidad del método implementado para el genotipado, y se identificaron las tres mutaciones, según se pudo corroborar al comparar los resultados obtenidos con las determinaciones hechas mediante el panel comercial StripAssay (Viennalab, Austria). El método descrito mostró la selectividad y la especificidad necesarias para la pesquisa clínica poblacional de la FQ, principalmente de la mutación F508del, dada su alta prevalencia entre la población cubana.

Palabras clave: Mutaciones en el gen CFTR, PCR en tiempo real alelo-específica, variación de Ct, fibrosis quística

*I*ntroduction

Mutations in the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene are associated with cystic fibrosis (CF), as the most common life-threatening autosomal recessive genetic disorder [1]. But also male infertility due to congenital bilateral absence of the vas deferens (CBAVD), idiopathic chronic pancreatitis and bronchiectasis are CFTR-related disorders. One mutation (F508del) accounts for about 70 % of CF alleles worldwide [2], and diverse heritages are reflected for the CFTR gene and distributed with varying frequencies among populations often complicating genetic analysis. Worldwide, most mutations are rare, with frequencies under 0.1 %, and just four mutations have relative frequencies of 1.2-2.4 % [3]. Regardless the frequency, F508del is always screened when testing for CFTR mutations [4].

Different detection methods have been implemented to detect the CFTR gene, among them: single-strand conformation polymorphism analysis (SSCP) [5], restriction fragment length polymorphism analysis, amplification refractory mutation system (ARMS) and real-time PCR systems using SYBR Green [6]. Commonly, exon analysis using SSCP or denaturing gradient gel electrophoresis (DGGE) are performed as indirect detection methods, but they are very laborious and time-consuming techniques, making them incompatible for the screening of a large number of samples.

Among the systems developed, allele-specific PCR (ASPCR) is an advantageous strategy which combines amplification and detection, and the successfully amplified product directly indicates the occurrence of a particular allele when present. The use of ASPCR together with real-time PCR techniques allows monitoring template amplification, consequently improving the interpretation of PCR results [7]. A typical

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ASPCR assay uses a specific primer to detect both the wild type (*wt*) and the mutant (*mut*) DNA sequences, and a common reverse primer. By these means, individuals can be genotyped by analyzing the resulting amplification products [8].

Theoretically, individuals without mutation generate a PCR product only in the wild type reaction, heterozygotes yields both the wild type and mutant reaction products, and homozygotes only in the mutant reaction. However, ASPCR involves a relative rather than an absolute phenomenon, hence, the mismatched template could also be amplified, although at much lower efficiency as compared with the matched one [9].

Since amplification of mismatched template occurs, the genotype was determined by comparing the Ct values (the number of PCR cycles at which reporter fluorescence becomes significant or is distinguishable from the background noise at a given threshold) of the sample in two parallel ASPCRs (*wt* and *mut*). The difference in Ct (ΔCt) could be used to predict the assay selectivity [10]. Similar Ct values could imply that both alleles, *wt* and *mut*, were present in the sample. There could be predicted the only allele (*wt* or *mut*) present in a sample just by comparing their Ct values, indicating a non-carrier or homozygous individual, depending on the earliest curve corresponding to the lower Ct. To attain such results, genomic DNA (gDNA) templates should be kept as homogeneous as possible to avoid variations between tubes corresponding to the same sample, which lead to false-positive results produced by the uneven distribution of different alleles of the same gene in the sample [11].

Based on these assumptions, here we describe the development of a fast and cost-effective method to detect three frequent CFTR mutations in Cuban population using ASPCR: F508del (c.1521-1523delCTT), 3120+1G>A (c.2988+1G>A) and N1303K (c.3909C>G). These three mutations change the polypeptide sequence of *CFTR*: phenylalanine is deleted at position 508, glycine is replaced by alanine at the first nucleotide in the intron following nucleotide 3120 and asparagine at position 1303 is replaced by lysine respectively [12]. The selectivity and specificity of the allele-specific real time PCR was confirmed through the cycle threshold variation (ΔCt) between wild type and mutant PCR products. The described method also provides the necessary selectivity and specificity required for the analysis of clinical samples. Furthermore, it reduces the possibilities of sample cross-contamination by avoiding post-hybridization reactions and it is easily reproducible by real-time PCR technology, with potential for the detection of other CFTR mutations.

Materials and methods

Primers design

Primers were designed with their 3´ nucleotide overlapping the polymorphic residue. Three primers per target were designed, one pair of primers targeting the mutant or a wild type allele, respectively, using the default parameters as recommended for allele discrimination, and a common primer by following the same recommendations as those for allele-specific primers. The 3´ terminal base of each allele-specific primer was adapted according to its corresponding mutation. Primers sequences for the detection of 3120+1G>A and N1303K polymorphisms were designed at the Center of Immunoassay using the *CFTR* sequence data available on the Cystic Fibrosis Mutation Database [13]. Primer sequences for detection of F508del were taken from Ferrie *et al*. [14]. Primer sequences are described in Table 1.

DNA extraction

Human gDNA samples from nine CF patients carrying the most frequent mutations reported in Cuba were kindly provided by the National Center of Medical Genetics (CNGM, Cuba) [15]. The gDNA was isolated from 10 mL of peripheral blood treated with ethylenediaminetetraacetic acid (EDTA) by salting out and samples were subsequently stored at –20 °C until use. Prior to the analysis, the gDNA quality and concentration was spectrophotometrically determined (optical density at 260; OD_{260nm}), its final concentration in the range of 5-40 μg/mL.

Genotyping

Genotype was previously characterized in all the samples by using the commercial panel StripAssay 4-410 (Viennalab, Austria). Five out of nine samples tested were single heterozygous (only one mutation detected): G85E, I507del, R553X, 3120+1G>A and R1162X, three as compound heterozygous (two mutations detected): R334W/F508del, G542X/F508del and N1303K/F508del; and one homozygous, F508del/F508del.

Real-time PCR assays

Three CFTR mutations were evaluated (F508del, 3120+1G>A and N1303K). Two PCR reactions per sample were run for every target (*wt* and *mut*). The *wt* allele-specific primer (ASP) of the corresponding target was added to one reaction and the *mut* ASP to the other. The common reverse primer was added to both reactions (*wt* and *mut*). Reactions were run in triplicate. The *wt* amplification curve was used as reference for the *mut* one and viceversa. All reactions were run in a reaction volume of 25 μL in 96-well plates with adhesive films and by using the SLAN 96-P real-time

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Table 1. Primer sets used for detecting the CFTR gene mutations by using allele-specific real time PCR

CFTR mutation	Sequence (5´-3´)*	Description	Amplicon length (bp)
F508del[¶]	GTATCTATATTCATCATAGGAAACACCATT	antisense wild type	160
	GTATCTATATTCATCATAGGAAACACCA<u>CA</u>	antisense mutant	
	GACTTCACTTCTAATGATGATTATGGGAGA	sense (common primer)	
3120+1G>A	CCTCTTACCATATTTGACTTCATCCACG	sense wild type	197
	CCTCTTACCATATTTGACTTCATCCAC<u>A</u>	sense mutant	
	AATTTACTAAACTTATGTCTATTTTGAAGGC	antisense (common primer)	
N1303K	GATCACTCCACTGTTCATAGGGATCCAAC	antisense wild type	206
	GATCACTCCACTGTTCATAGGGATCCAA<u>G</u>	antisense mutant	
	GAGAGAACTTGATGGTAAGTACATGGGTGTTTC	sense (common primer)	

* Letters corresponding to mutated nucleotides are underlined.
[¶] Primer set taken from: Ferrie RM, Schwarz MJ, Robertson NH, Vaudin S, Super M, Malone G, *et al*. Development, multiplexing and application of ARMS tests for common mutations in the CFTR gene. Am J Hum Genet. 1992;51:251-62.

PCR system (Shanghai Honshi Medical Technology Co., Ltd; China). Each reaction mixture contained 1× Absolute qPCR SYBR Green Mix (Thermo Scientific, Liege, Belgium). During the optimization steps, primer concentrations were set to 0.3 µM; the volume was completed to 20 µL with DNase-free water and 5 µL of template gDNA (25-200 ng) was added for each sample. The thermal PCR profile was: 15 min at 95 °C for Thermo Start DNA Polymerase activation, followed by 38 amplification cycles (95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s), and a final extension step at 72 °C for 3 min.

The test threshold was set at 0.12. The average Ct values for the three replicas were determined and ΔCt calculated for each sample: ΔCt = Average Ct (mutant ASO) – Average Ct (wild type ASP). High Resolution Melting (HRM) analysis was performed to evaluate the specificity of the mutant allele-specific primers. PCR products were melted by increasing the temperature from 60 to 90 °C at a programmed rate of 0.1 °C/s, and melting curves analyzed with the aid of the commercial SLAN 96-P software, version 8.2 (Shanghai Honshi Medical Technology Co., Ltd; China).

Results

ASPCR reactions were performed to evaluate if the experimental conditions were capable to discriminate samples containing the mutations variants F508del, 3120+1G> or N1303K of CFTR, the typical amplification curves of ASPCRs containing *wt* or *mut* ASP targeting mutations are shown in figures 1 to 3, respectively.

In the case of target F508del (Figure 1A), the amplification product in the reaction containing the *mut* ASP (red) was detected significantly earlier than that for the *wt* reaction, in the homozygous F508del sample. The similar accumulation of both ASPCR products allows identifying the heterozygote F508del sample (Figure 1B). Moreover, non-carrier F508del genotypes were identified (Figure 1C), inversely as compared with that previously seen for the homozygous sample, the amplification product in the *wt* ASP reaction been detected significantly earlier than the *mut* product. The same interpretation was given to 3120+1G>A and N1303K targets profiles shown in figures 2 and 3, respectively. Data resulting from PCR analysis of the three mutations analyzed are shown in tables 2 to 4.

The ΔCt values were widely separated to accurately determine the non-carrier and homozygoous genotypes in every sample. Similar ΔCt values were obtained for all heterozygous samples ($-1 \leq \Delta Ct \leq 1$). The ΔCt ranges for non-carriers of each mutation (mean value ± SD) were: 7.4 to 8.1 for F508del; 10.26 to 11.16 for 3120+1G>A; and 9.58 to 9.98 for N1303K. On the contrary, heterozygous carriers developed ΔCt ranges as follows: F508del, –0.20 to –0.36; 3120+1G>A, –0.14 to –0.18; and N1303K, –0.14 to –0.18. Homozygous samples (F508del) showed a mean ΔCt value of –12.13 ± 0.45, confirming the reliable detection reported for this frequent genotype (see tables 2 to 4).

All heterozygous samples demonstrated ΔCt values lower than 1 cycle, whereas non-carriers samples showed ΔCt > 7 values, the homozygous showing ΔCt < –11 (negative values). The specificity of ASPCR was verified using the derivative melting curve. As shown in figure 4, the amplification reactions were specific since only one peek per target was obtained. The melting temperatures of the generated amplicons were 80.0 °C (F508del), 77.55 °C (3120+1G>A) and 76.44 °C (N1303K).

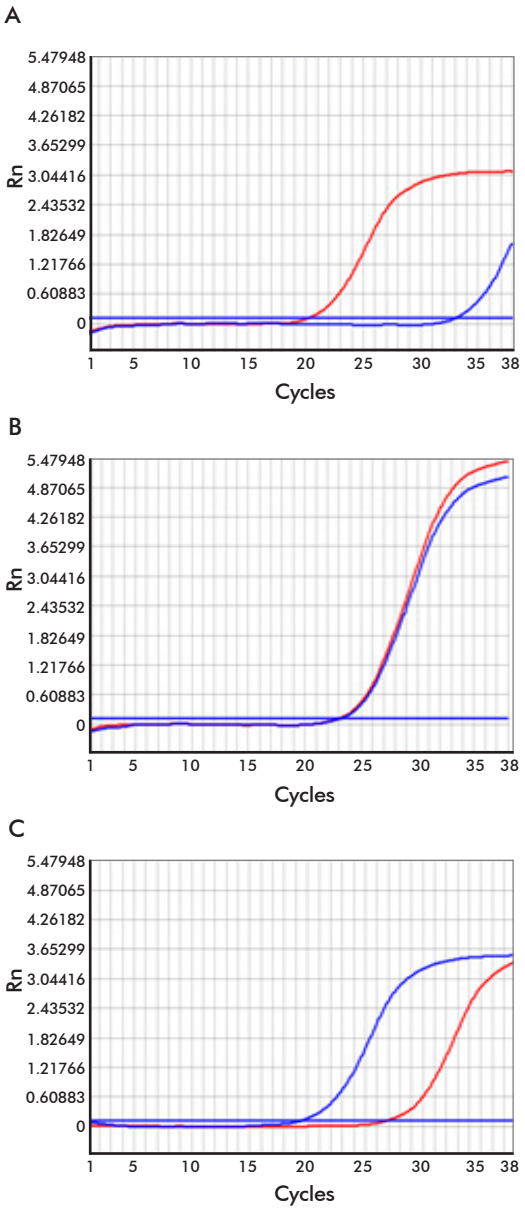

Figure 1. Amplification curves obtained after ASPCR targeting for F508del mutation (c.1521-1523delCTT) of the *CFTR* gene of samples from cystic fibrosis patients. A) Sample homozygous for the F508del mutation. B) Sample of a heterozygous carrier of the F508del mutation. C) Non-carrier sample. The mutant (*mut*) ASPCR product is depicted in red and the wild type (*wt*) product in blue.

Discussion

Studies based on ASPCR have been developed for clinical research or testing of the IVS8 (TG)

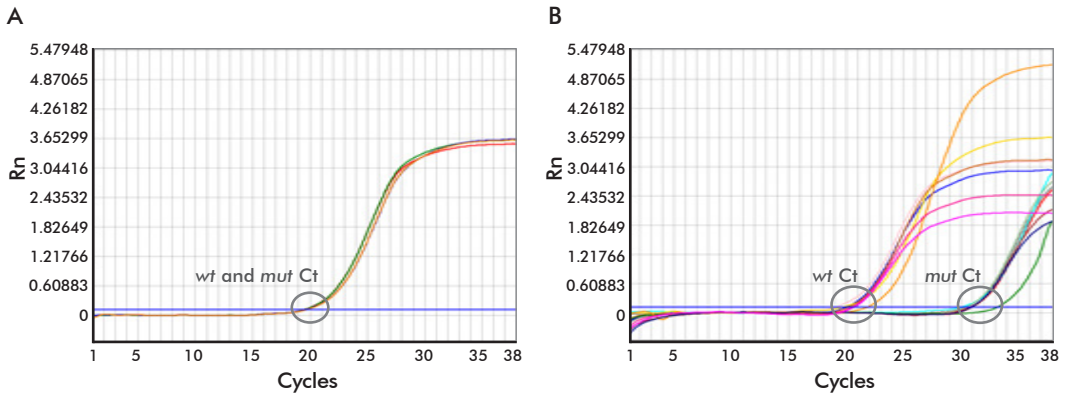

Figure 2. Amplification curves obtained after ASPCR targeting the 3120+1G>A (c.2988+1G>A) mutation of the CFTR gene in samples from cystic fibrosis patients. A) ASPCR profile of positive samples carrying one 3120+1G>A allele, exhibiting similar Ct values for the mutant (*mt*) and wild type (*wt*) curves. Two samples were tested (two runs per sample). B) ASPCR profile of samples from non-carriers widely separated Ct values for the *wt* and *mut* curves. Seven samples were tested (two runs per sample) Samples corresponding to the target and the F508del homozygous mutations were excluded, due to the their similar Ct values.

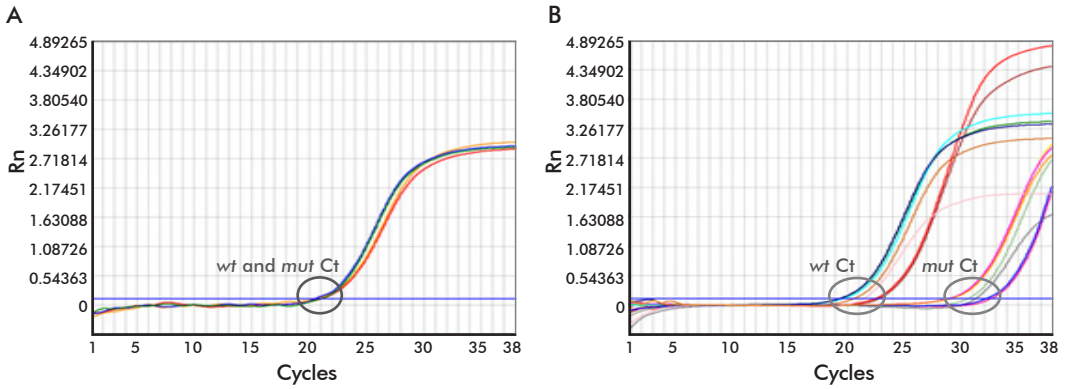

Figure 3. Amplification curves obtained after ASPCR targeting the N1303K (c.3909C>G) mutation of the CFTR gene in samples from cystic fibrosis patients. A) ASPCR profile of samples carrying one N1303K (c.3909C>G) allele, exhibiting similar Ct values for the mutant (*mt*) and wild type (*wt*) curves. B) ASPCR profile of samples from non-carriers widely separated Ct values for the wt and mut curves. Seven samples were tested (two runs per sample). Samples corresponding to the target and the F508del homozygous mutations were excluded, due to the their similar Ct values.

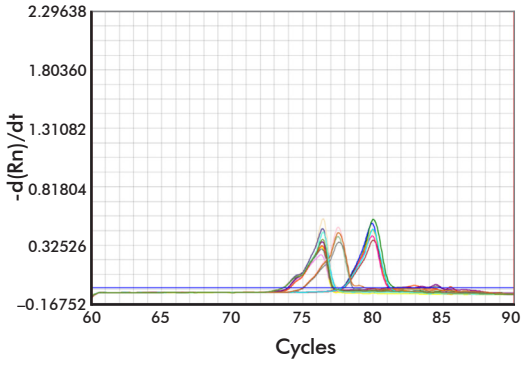

Figure 4. High Resolution Melting (HRM) analysis of three mutant ASPCR products for *CFTR* gene mutations N1303K, 3120+1G>A and F508del. The melting temperatures of the products were 80.0 °C (F508del), 77.55 °C (3120+1G>A) and 76.44 °C (N1303K). An adequate numbers of replicas were tested for each mutation.

m(T)n locus in the *CFTR* gene [16], common mutations in *KRAS* and *BRAF* [7] and hepatitis B virus-resistant strains [17]. This study shows the development of a highly specific ASPCR method targeting three of the most common *CFTR* mutations (F508del, 3120+1G>A and N1303K) in the same IVS8 (TG)m(T)n locus among the Cuban population.

ASPCR is based on *Taq* polymerase lacking 3′ to 5′ exonuclease activity, the resulting mismatch between the 3′ end of the PCR primer and the template leading to highly reduced amplification efficiency [9]. As expected, a difference in the ΔCt values between the *wt* and *mut* ASPCR was high enough to correctly discriminate the presence of *wt* from the *mut* allele for the three mutations tested. This is in agreement with control determinations by using commercial panel StripAssay (Viennalab, Austria), with neither false positive nor doubtful result obtained.

Since CFTR mutations are variations at the germline level, individuals can be non-carriers (mutation absent), heterozygous (50 % frequency, in a single allele) or homozygous (100 %). For these reasons, genotyping assays just require 50 % selectivity; that is, assays must be able to detect one mutant template in the presence of one wild type template

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[9]. In this sense, 50 % selectivity was achieved for two targets (3120+1G>A and N1303K) and 100 % for F508del due to its homozygocity, in fact, the only one homozygous mutation. Homozygous combinations are rare [18], and neither 3120+1G>A nor N1303K homozygocity has not been reported in Cuba to date. But, significantly, the method developed is designed to be able to detect them in case that they would appear among the screened populations.

Regarding ΔCt values, Ct is sensitive to the initial amount of templates. If initial gDNA concentrations were too low, the first curve could emerge after 30 cycles and the second curve could not be visible, possibly making the analysis unreliable. So far, we established the reproducibility of the assay in the range of 25-200 ng of gDNA to avoid the displacement of the amplification curves to left or right, which could generate misleading data, in spite of not determining the sensitivity for these assay. Therefore, further studies will be conducted to establish the limits of sensitivity (*i.e.*, lowest amount) of gDNA needed to attained reproducible results. This element is relevant for the implementation of high throughput screening and simultaneous determinations from single samples.

Finally, the specificity of these assays was demonstrated, with not any unspecific PCR products been amplified when samples carrying other related *CFTR* mutations were tested. The HRM analysis of mutant ASPCRs confirmed the presence of specific PCR products when at least one of the alleles in the sample was affected (Figure 4). The derivative melting curves showed only one peak, indicating a single amplified target on each reaction. Since HRM analysis was performed, therefore, a post-PCR melting analysis was not required. Nevertheless, Nagy *et al*. [19] suggested that a post-PCR melting analysis could complement data and lead to more accurate results, aiding to detect possible unspecific amplification signals. Using HRM data only, all heterozygotes and most homozygotes could be detected without mixing [20], although F508del is sometimes not distinguished from wild type [21]. The ΔCt values analysis used in this work solved this problem; therefore, avoiding the need for complementary analysis such as HRM to detect mutations.

Overall, the method developed provides some valuable advantages as compared with other methodologies. These assays do not require internal amplification controls. If no product is generated in the allele-specific reaction this can be due to the presence of inhibitors in the sample. Both amplification curves, *wt* and *mut*, should always be present. Our ASPCR-based method loses its reliability when both reactions from the same sample are not carried out at the same time. The assays should also be performed in a calibrated PCR system to avoid variations especially among tubes or wells.

Another relevant feature of this assay is that interpretation of the results is based on ΔCt differences between two reactions, easy to visualize and does not require additional software or the use of any complex algorithms for results discrimination. The method described herein allows fast confirmation (in less than 2 h) as compared with other more laborious standard procedures, of three *CFTR* mutations, and not requiring solid-phase hybridization or any other expensive sequencing process. Moreover, additional contamination risks are minimized since there is no need for additional processing after PCR, the samples never leaving the amplification plate.

Regarding CF, the identification of F508del and its association with the clinical severity of the disease supports a better understanding of its influence on clinical manifestations of the disease [22]. In fact, considering the high prevalence of the F508del mutation [12] and the advantages of the developed method, we could recommend to implement

Table 2. Evaluation of cystic fibrosis mutations samples using ASPCR targeting the F508del mutation in samples of 9 genotyped patients

Patient's sample code*	Genotype	Average Ct ASPCR (*wt*)	Average Ct ASPCR (*mut*)	ΔCt
A1	G85E	20.09	28.02	7.93
A2	R334W/F508del	23.19	22.97	–0.22
A3	I507del	21.05	29.12	8.07
A4	G542X/F508del	23.17	22.79	–0.38
A5	R553X	19.41	26.82	7.41
A6	3120+1G>A	19.27	26.68	7.41
A7	R1162X	21.27	29.14	7.87
A8	N1303K/F508del	21.15	20.90	–0.25
A9a	F508del/F508del	32.62	20.16	–12.46
A9b	F508del/F508del	32.04	20.23	–11.81

* Different letters for the same sample represent runs of the sample on different days, to verify the detection of the target mutation.

Table 3. Evaluation of cystic fibrosis mutations samples using ASPCR targeting the 3120+1G>A mutation in samples of 9 genotyped patients

Patient's sample code*	Genotype	Average Ct ASPCR (*wt*)	Average Ct ASPCR (*mut*)	ΔCt
B1	G85E	20.01	30.85	10.84
B2	R334W/F508del	21.87	33.23	11.36
B3	I507del	20.51	30.99	10.48
B4	G542X/F508del	22.02	32.97	10.95
B5	R553X	19.68	30.04	10.36
B6a	3120+1G>A	19.65	19.51	-0.14
B6b	3120+1G>A	19.71	19.53	-0.18
B7	R1162X	20.71	30.89	10.18
B8	N1303K/F508del	19.15	30.40	11.25
B9	F508del/F508del	20.20	30.49	10.29

* Different letters for the same sample represent runs of the sample on different days, to verify the detection of the target mutation.

Table 4. Evaluation of cystic fibrosis mutations samples using ASPCR targeting the N1303K mutation in samples of 9 genotyped patients

Patient's sample code*	Genotype	Average Ct ASPCR (*wt*)	Average Ct ASPCR (*mut*)	ΔCt
C1	G85E	20.44	30.22	9.78
C2	R334W/F508del	22.46	32.17	9.71
C3	I507del	19.40	28.99	9.59
C4	G542X/F508del	22.80	32.60	9.80
C5	R553X	19.62	28.98	9.36
C6	3120+1G>A	19.35	28.84	9.49
C7	R1162X	21.40	31.22	9.82
C8	F508del/F508del	20.57	30.56	9.99
C9a	N1303K/F508del	20.52	20.97	0.45
C9b	N1303K/F508del	21.58	21.60	0.02
C9b	N1303K/F508del	21.02	20.50	–0.52

* Different letters for the same sample represent runs of the sample on different days to verify the detection of the target mutation. The N1303K sample corresponds to the only confirmed individual carrying this mutation among the Cuban population.

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it as the primary massive screening tool, mainly in prenatal diagnosis and also for carrier screening. Due to the low prevalence of 3120+1G>A and N1303K, these methods should be used for confirmation after previous exon scanning techniques like DGGE or SSCP.

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