Am. J. Hum. Genet. 51:251–262, 1992

Development, Multiplexing, and Application of ARMS Tests for Common Mutations in the CFTR Gene

Richard M. Ferrie,* Martin J. Schwarz,† Nancy H. Robertson,* Sally Vaudin,* Maurice Super,† Geraldine Malone,† and Stephen Little*

*Cellmark Diagnostics, Abingdon, Oxfordshire; and †Paediatric Genetics Unit, Royal Manchester Children's Hospital, Manchester, England

Summary

The amplification refractory mutation system (ARMS) is a simple, rapid, and reliable method for the detection of any mutation involving single base changes or small deletions. We have applied ARMS methodology to the detection of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Single ARMS tests have been developed for 11 CFTR mutations found in the northwest of England. ARMS reactions for the most common mutations have been multiplexed to give a test which will detect the presence of the ΔF508, G551D, G542X, and 621 + 1G→T mutations in a DNA sample. The multiplex test has been validated by the analysis of over 500 previously genotyped samples and has been found to be completely accurate. The rapid detection of the most common mutations has enabled early molecular confirmation of suspected cystic fibrosis in neonates, rapid typing of cystic fibrosis patients and their relatives, and testing of sperm and egg donors.

Introduction

The ability to detect changes in DNA sequence is of great importance in the field of medical genetics. Traditionally, detection of DNA variation has been performed by analysis of RFLPs by using the Southern blotting technique (Southern 1975; Kan and Dozy 1978; Wyman and White 1980). As this approach is relatively slow and technically demanding, new methods based on the PCR (Saiki et al. 1985) have been devised. Several adaptations of the PCR have been described for the detection of DNA variation. These include RFLP analysis (Chehab et al. 1987), the creation of artificial RFLPs by the use of primer-specified restriction-map modification (Haliassos et al. 1989), hybridization to allele-specific oligonucleotides (ASOs) (Saiki et al. 1986), or detection of small deletions by determination of the size of the PCR product (Rommens et al. 1990). Of these methods, only the ASO approach can be applied to any point mutation or small deletion, as the others are dependent on the nature of the mutation and the surrounding DNA sequence. The ASO approach has the disadvantage that the oligonucleotides or the PCR products must be labeled.

Recently, Newton et al. (1989) described the amplification refractory mutation system (ARMS) as a general technique for the analysis of *any* point mutation or small deletion. The basis of the system is the observation that oligonucleotides which are complementary to a given DNA sequence except for a mismatch at their 3′ OH residue will not function as primers in the PCR under appropriate conditions. A typical ARMS test consists of two complementary reactions. The first reaction contains an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus. Similarly, the second reaction contains a mutant-specific primer and does not amplify normal DNA. The genotype of an individual can be determined by analysis of the amplification products. A normal individual generates PCR product only in the normal reaction; a heterozygote gives products in both reactions, and a homozygous mutant individual does so only in the mutant reaction.

The ARMS system has several advantages over other PCR-based analysis systems. The method is rapid, reliable, and nonisotopic, and results can be

Received January 22, 1992; revision received March 10, 1992.

Address for correspondence and reprints: S. Little, ICI Cellmark Diagnostics, Gadbrook Park, Northwich, Cheshire CW9 7RA, England.

0002-9297/92/5102-0004$02.00

easily obtained in one working day. The use of two reactions with internal controls ensures that false-negative results are not obtained. In principle, ARMS tests can be developed for any mutation, and ARMS has already been applied to the detection of several genetic polymorphisms. These include α1-antitrypsin deficiency (Newton et al. 1989), sickle cell anemia (Wu et al. 1989), phenylketonuria (Sommer et al. 1989), cystic fibrosis (CF) (Ballabio et al. 1990; Wagner et al. 1990), apoliprotein E (Wenham et al. 1991), β-thalassaemia (Old et al. 1990), and internal variation within the D1S8 locus (Jeffreys et al. 1991).

It is now becoming clear that, for many genetic diseases, there is more than one mutation responsible for the condition. Furthermore, the mutations can be closely spaced, often within a few base pairs of each other. Examples of such multimutational diseases include CF (Cutting et al. 1990), β-thalassaemia (Old et al. 1990), and Tay-Sachs disease (Myerowitz 1988). This makes mutation analysis more complex, and, clearly, a system which could simultaneously analyze a sample for the presence of multiple mutations would be useful.

The aim of this study was to apply the ARMS system to the simultaneous analysis of several mutations within the CF transmembrane regulator (CFTR) gene (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989). Since the discovery that ΔF508 was the major CF mutation, over 150 further CF mutations have been reported to the Cystic Fibrosis Genetic Analysis Consortium. Although it is often desirable to analyze samples for as many CF mutations as possible, there are practical limits to the number which can routinely be performed. Here we demonstrate that multiplex ARMS allows the reliable detection of CF mutations and reduces the time and effort required for DNA analysis.

The approach we adopted was to divide the study into four separate sections; these were (i) identification of CF mutations in the population in the northwest of England, (ii) development of individual ARMS reactions for the common mutations, (iii) multiplexing of the tests for the most common mutations, and (iv) validation and clinical application of the resulting multiplex test.

Methods

DNA Extraction

DNA was extracted from blood or buccal cells in one of three ways. The first was a modification of the method of Kunkel et al. (1977) and required 5–10 ml of blood. The second was a micropreparative method; 800 μl of NH_4Cl (170 mM) was added to 200 μl of thawed EDTA blood and was mixed for 20 min by rotating. The mixture was centrifuged, and the resultant cell pellet was washed three times with 300 μl of wash buffer (10 mM NaCl/10 mM EDTA). The pellet was resuspended in 500 μl of 50 mM NaOH and boiled for 20 min. Following neutralization with 100 μl of 1M Tris-HCl (pH 7.5), the pellet was centrifuged briefly to remove the cell debris, and 5 μl of the resulting supernatant DNA solution was used in each ARMS reaction. The third method employed mouthwash samples derived from the agitation of 10 ml of 4% sucrose solution in the mouth for 20 s (Lench et al. 1988). The suspension was centrifuged at 1,200 *g* for 10 min and was washed in 500 μl of wash buffer. After resuspension in 500 μl of 50 mM NaOH, the sample was processed as in the second method.

ARMS Reaction Conditions

The standard ARMS reaction mixture contained 43 pmol of each of the relevant primers (made on an Applied Biosystems model 340A synthesizer) and 4.3 nmol of dNTP (dATP, dCTP, dGTP, and TTP; Pharmacia) in reaction buffer (43 mM KCl, 8.6 mM Tris-HCl [pH 8.3], 1.0 mM $MgCl_2$, and 0.008% gelatin). The reaction mixture (45 μl) containing all components except enzyme were heated to 94°C for 5 min in a Perkin Elmer Cetus DNA thermal cycler. Following the addition of enzyme (1 unit of Cetus *Taq* DNA polymerase in 5 μl of reaction buffer), 35 cycles of PCR were performed under the following conditions: 94°C denaturation for 2 min, 60°C annealing for 2 min, and 72°C extension for 2 min. The procedure was completed by the continuation of the last 72°C incubation for a further 10 min. Electrophoresis of the products of the ARMS reaction was carried out in a 3% (3:1 agarose:Nusieve) gel containing ethidium bromide and was visualized under UV light.

Results

CF Mutations in the Northwest of England Population

DNA samples from CF affected individuals and their relatives were screened in order to determine the frequency of CF mutations in the northwest of England. Three different PCR-based techniques were used in this initial study—amplicon size difference, RFLPs, and ASO hybridization. The mutations detected, their frequencies, and the methods used to

Table 1

CF Mutations in 1,030 Chromosomes from the Northwest of England

Mutation	Observed Frequency (%)	Detection Method[a]	Reference
R117H	.4	A	Dean et al. 1990
621 + 1G>T ...	1.0	R	Zielenski et al. 1991
ΔI507	.5	S	Schwartz et al. 1991
ΔF508	80.7	S and A	Kerem et al. 1989
1717-1G>A ...	.3	A	Guillermit et al. 1990
G542X	1.1	A	Kerem et al. 1990
G551D	3.4	R	Cutting et al. 1990
R553X	.8	R	Cutting et al. 1990
R560T	.6	R	Kerem et al. 1990
W1282X	.2	A	Vidaud et al. 1990
N1303K	.6	A	Osborne et al. 1991

[a] A = ASO hybridization; R = RFLP; and S = size difference.

identify them are summarized in table 1. In total, 11 mutations were observed, accounting for 91.5% of all CF chromosomes in the population. Although the techniques used to generate these data were reliable, they were also slow and technically demanding. To facilitate further analysis, ARMS tests were developed for the 11 mutations.

Development of ARMS Tests for Single CF Mutations

Normal and mutant-specific ARMS primers were designed such that their terminal 3′ base was homologous to either the normal or altered nucleotide of the mutation under study. Under the standard ARMS conditions it was generally necessary to introduce additional deliberate mismatches in order to improve discrimination between normal and mutant alleles. Our initial approach was to introduce these extra mismatches adjacent to the terminal 3′ base, although in a few cases the effect of mismatches at the − 3 position was also studied.

ARMS tests were developed for the following mutations: ΔF508 (Riordan et al. 1989), ΔI507 (Schwarz et al. 1991), R117H (Dean et al. 1990), 621 + 1G→T (Zielenski et al. 1991), 1717G→A (Guillermit et al. 1990), W1282X (Vidaud et al. 1990), G551D and R553X (Cutting et al. 1990), G542X and R560T (Kerem et al. 1990), and N1303K (Osborne et al. 1991). Unless otherwise specified, the temperature cycling parameters of the PCR reaction, the buffer conditions, and the concentration of the ARMS primers were kept constant. The finalized versions of the single ARMS tests all included an internal control for the PCR reaction. These were either control primers AAT1/2 (220-bp fragment of exon V of α1-antitrypsin gene) or AAT3/4 (360-bp fragment of exon III) (Newton et al. 1989).

Note on Primer Nomenclature

A large number of oligonucleotide PCR primers (amplimers) were used in this study. Each primer has been given a three-part code of the form XX-y-Z. The first part refers to the CF mutation under study; for example, R117H is abbreviated to RH and G551D to GD. The second part ("y" in the example above) indicates which additional mismatches (if any) have been included at the penultimate base of the amplimer. Each mismatch has a specific code given in table 2. If any additional mismatch is included at a position other than the penultimate base, this is indicated by the inclusion of the base number after the mismatch code. The third part of the code indicates whether the primer is specific for the mutant sequence (M), specific to the normal sequence (N), or common to both (C). Most of the ARMS primers were complementary to the antisense strand of the CFTR gene. In some cases primers complementary to the sense strand were used, and this is indicated by the replacement of the − symbol with a + symbol in ARMS and common primers codes; for example, RH-d-N indicates that this primer is used to analyze the R117H mutation (RH), that it has an A residue as the penultimate base which mismatches a C residue in the target sequence (code d), and that it is specific for the normal allele (N). The bases in the primers are numbered from the 3′ end; thus the 3′ terminal base is − 1, the penultimate base is − 2, etc.

ΔF508 and ΔI507

These two adjacent mutations differ from others in this study in that they are deletions rather than

Table 2

Mismatch Codes

Primer Sequence[a]	Template Sequence[b]			
	A	C	G	T
A	b	d	e	
C	f	h		j
G	k		q	r
T		s	v	w

[a] Bases in ARMS primer.
[b] Bases in target DNA.

substitutions. The development of these ARMS tests was complicated by the existence of two additional benign mutations of the CFTR gene, namely F508C and I506V (Kobayashi et al. 1990). The ARMS primers described by Newton et al. (1989) do not allow ΔF508 and ΔI507 to be discriminated from these benign variants. To overcome this problem, two separate ARMS tests were devised. The first test used primers DF-j-N and DF-w-M and was ΔF508 specific. In this test the mutant ARMS primers are complementary to sequences distal to ΔF508 and hence do not recognize the ΔI507 mutation. This is illustrated in figure 1. DNA samples carrying the benign mutations were not available for analysis, but, as the I506V mutation was proximal to the ARMS target site, and as the F508C mutation was complementary to the normal ARMS primer, it seems likely that samples containing either of these sequences would appear normal in this test.

A second ARMS test, using primers complementary to sequences proximal to DF508 (IF + N and IF + b5 + M), was also developed. This test identified both the ΔF508 and ΔI507 mutations (fig. 1). By using both tests in combination, ΔI507 and ΔF508 mutations could be identified and discriminated. Primers AAT3 and AAT4 were included as an internal control for the PCR reaction. The sequences of the ARMS primers used are listed in table 3.

R117H

The R117H mutation of exon IV of the CF gene is a G→A substitution at position 482 (Dean et al. 1990). The two ARMS primers initially designed (RH-d-N and RH-d-M; fig. 2) incorporated an additional C/A mismatch at position −2 and generated a 237-bp product when used in combination with the common primer RH-C. However, when they were used at the standard concentration of 1 μM, the presence of mutant ARMS product in the DNA from a normal individual indicated that the mutant primer was not specific (fig. 2*a*). An R117H homozygote was not available for us to confirm the specificity of the normal primer.

Three approaches were used in order to design an ARMS test specific for the R117H mutation. They were as follows:

1. *Reduction to the concentration of ARMS primers.* — When the concentration of mutant ARMS primers was reduced from the standard concentration of 0.86 μM to 0.10 μM, the reaction was mutant specific (fig. 2*b*), although the yield of mutant R117H ARMS product was slightly reduced.

2. *Inclusion of a control PCR reaction.* — Specificity of the mutant ARMS primer RH-d-M could also be achieved at the standard concentration, providing that an additional PCR reaction was performed in the same tube. When control primers AAT3 and AAT4 (product size 360 bp) were included in both R117H ARMS reactions at a concentration of 0.86 μM, it was noted that the faint mutant R117H band previously detected in normal individuals was no longer visible (fig. 2*c*). In ARMS tests of individuals heterozygous for the R117H mutation, the intensity of normal and mutant R117H ARMS product bands was equal.

3. *Additional 3′ mismatch destabilization in ARMS primers.* — Two further sets of R117H ARMS primers were designed. In these primers, the mismatch present at position −2 at the 3′ end of the primers was varied in order to destabilize the interaction between the primers and template DNA. Primers RH-s-N and RH-s-M gave a C/T mismatch, and RH-h-N and RH-h-M gave a C/C mismatch. At the standard concentration, the C/T mismatch was found to be less destabilizing than the C/C mismatch, as judged by yield of mutant ARMS product from normal DNA. The use of the primers incorporating the C/C mismatch resulted in a specific R117H test (fig. 2*d*).

Single ARMS tests for Eight Further CF Mutations

Single ARMS tests for an additional eight mutations were designed using the optimization strategies discussed above, in the section on R117H. The mutations for which ARMS tests were developed were R560T, R553X, G551D, G542X, 1717-1G→A, 621 + 1G→T, N1303K, and W1282X. The primers selected for each mutation are detailed in table 3, and examples of these tests in use can be seen in figure 3. As all of the tests developed included an internal control PCR reaction, a failure of PCR in one reaction tube would not result in misdiagnosis.

The Development of a Multiplex ARMS Test for the Four Most Common CF Mutations in the Northwest of England

In order to obtain a multiplex ARMS test for these four mutations (ΔF508, G551D, G542X, and 621 + 1G→T; table 1), it was necessary to develop a method for the simultaneous detection of the two closely linked exon XI mutations, G551D and G542X, which are separated by only 28 bp. The method devised (overARMS) works by employing the same common

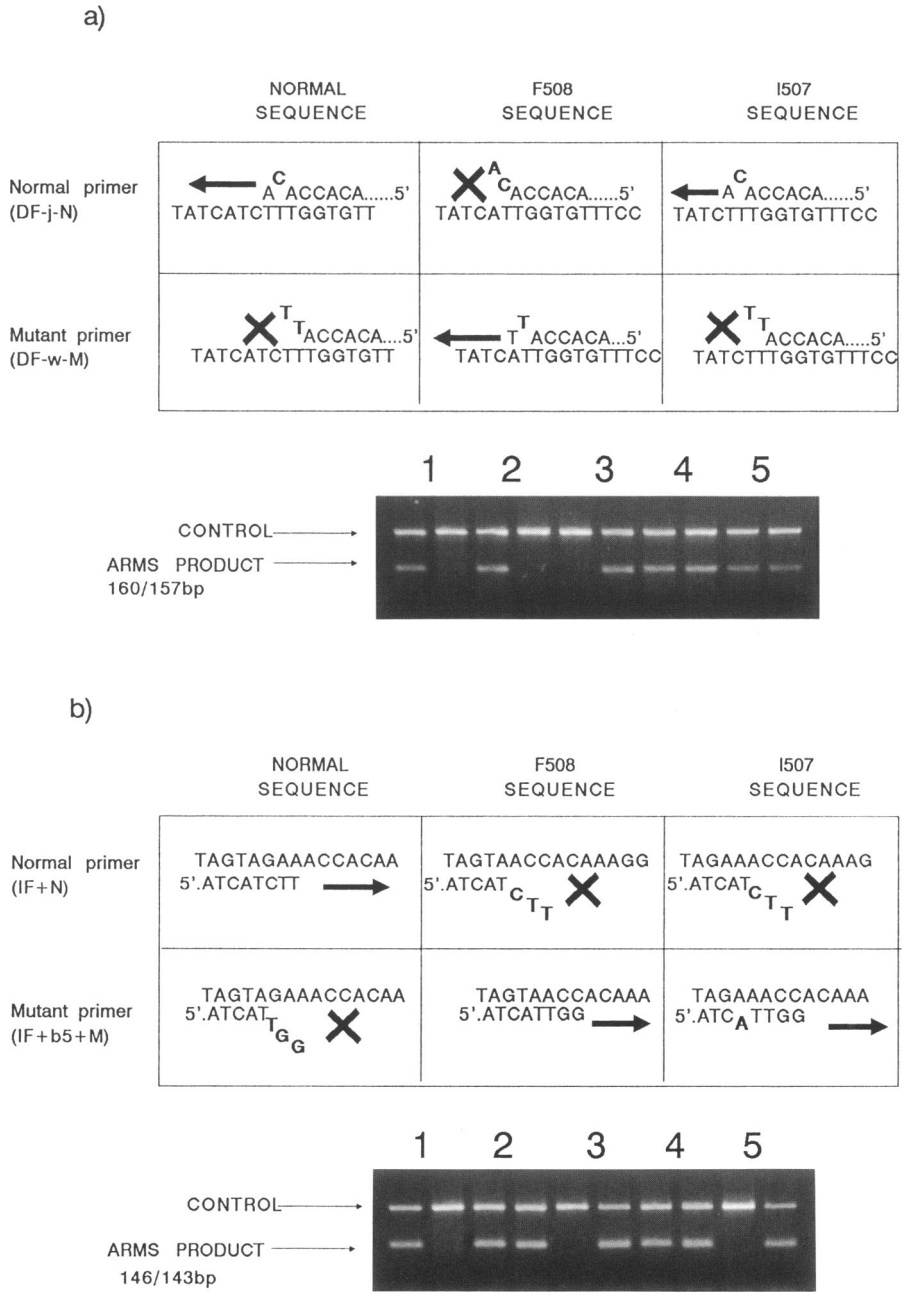

Figure 1 *a*, Sequences of the ARMS primers and the target DNA in the ΔF508-specific ARMS test. The diagrams in the boxes align the normal and mutant ARMS primers (3′ to 5′) with the normal, ΔF508, and ΔI507 target DNA sequences. Complementary bases in the ARMS primers are shown in normal type, whereas noncomplementary bases are shown in boldface type and displaced from the target sequence. An arrow indicates primer/target combinations which act as a substrate for *Taq* DNA polymerase under standard ARMS conditions, and a cross shows where extension does not occur. Below the boxes are the results of an ARMS analysis using the ΔF508-specific primers (DF-j-N and DF-w-M). For each of the five samples there are two tracks; the first shows the products from the normal ARMS reaction, and the second shows those from the corresponding mutant reaction. *b*, Corresponding information for the ΔI507/ΔF508-specific primers (IF + N and IF + b5 + M). In this case the ARMS primer sequence is written 5′ to 3′. The genotypes of the five samples are normal (lane 1), ΔI507 heterozygote (lane 2), ΔF508 homozygote (lane 3), ΔF508 heterozygote (lane 4), and ΔF508,ΔI507 compound heterozygote (lane 5). These genotypes can be inferred from the combined results of both analyses.

Table 3

Sequences of Primers Used for the Single ARMS Tests

Mutation and Sequence (5′ to 3′)	Code	Size (bp)	AAT Primers
ΔF508:			
GACTTCACTTCTAATGATGATTATGGGAGA	DF-C		
GTATCTATATTCATCATAGGAAACACCACA	DF-j-N	160	AAT3 + 4
GTATCTATATTCATCATAGGAAACACCATT	DF-w-M	157	
ΔF508, ΔI507:			
GGGTAGTGTGAAGGGTTCATATGCATAATC	IF + C		
GCCTGGCACCATTAAAGAAAATATCATCTT	IF + N	146	AAT3 + 4
GCCTGGCACCATTAAAGAAAATATCATTGG	IF + b5 + M	143	
Exon 11:			
TAAAATTTCAGCAATGTTGTTTTTGACC	11-C		
R560T			
GCTTGCTAGACCAATAATTAGTTATTCATC	RT-v-N	317	AAT1 + 2
GCTTGCTAGACCAATAATTAGTTATTCATG	RT-v-M	317	
R553X:			
CACCTTGCTAAAGAAATTCTTGCTAG	RX-e-N	291	AAT1 + 2
CACCTTGCTAAAGAAATTCTTGCTAA	RX-e-M	291	. . .[a]
G551D:			
GCTAAAGAAATTCTTGCTCGTTGCC	GD-j-N	285	AAT1 + 2
AGCTAAAGAAATTCTTGCTCGTTGCT	GD-j-M	286	
G542X:			
ACTCAGTGTGATTCCACCTTCTAC	GX-e-N	256	AAT1 + 2
CACTCAGTGTGATTCCACCTTCTCA	GX-M	257	
1717-1G>A:			
GTCTTTCTCTGCAAACTTGGAGATGTGC	1717-q-N	220	AAT3 + 4
GTCTTTCTCTGCAAACTTGGAGATGTGT	1717-q-M	220	
R117H:			
CACATATGGTATGACCCTCTATATAAACTC	RH-C		
CCTATGCCTAGATAAATCGCGATAGAAC	RH-d-N	237	AAT3 + 4
CCTATGCCTAGATAAATCGCGATAGAAT	RH-d-M	237	
N1303K:[b]			
CTCAATTTCTTTATTCTAAAGACATTGG	NK-C		
GATCACTCCACTGTTCATAGGGATCCAAG	NK-N	328	AAT3 + 4
GATCACTCCACTGTTCATAGGGATCCAAC	NK-M	328	. . .[a]
621 + 1G>T:			
TCACATATGGTATGACCCTCTATATAAACT	621-C		
TGCCATGGGGCCTGTGCAAGGAAGTATTCC	621-j-N	380	AAT1 + 2
TGCCATGGGGCCTGTGCAAGGAAGTATTCA	621-j-M	380	
W1282X:			
CCCATCACTTTTACCTTATAGGTGGGCCTC	WX-C		
CCTGTGGTATCACTCCAAAGGCTTTCCAC	WX-b-N	178	AAT3 + 4
CCTGTGGTATCACTCCAAAGGCTTTCCAT	WX-b-M	178	
AAT:			
TGTCCACGTGAGCCTTGCTCGAGGCCTGGG	AAT1	220	
GAGACTTGGTATTTTGTTCAATCATTAAG	AAT2	220	
CCCACCTTCCCCTCTCTCCAGGCAAATGGG	AAT3	360	
GGGCCTCAGTCCCAACATGGCTAAGAGGTG	AAT4	360	

[a] Used at 0.2 × standard concentration.

[b] Used at 2.0 × standard concentration.

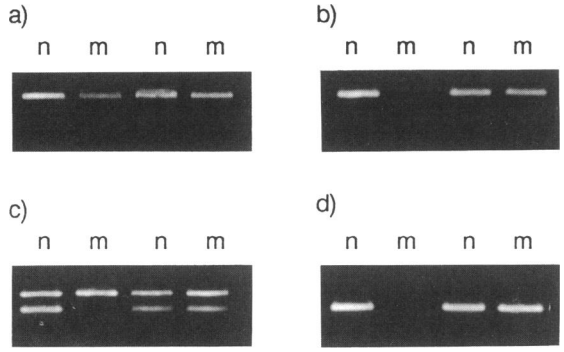

Figure 2 Development of a single ARMS test for R117H. The first two tracks in each panel are the products of the normal (lanes n) and mutant (lanes m) reactions using a normal DNA sample. The second pair of tracks in each panel are the products when DNA from an R117H heterozygote is used. Shown are the results obtained using primers RH-d-N and RH-d-M at 0.86 μM (*a*), at reduced primer concentration 0.10 μM (*b*), at 0.86 μM with the inclusion of AAT1/2 control primers (*c*), and using primers RH-h-N and RH-h-M (which are identical to RH-d-N/M, except for C/C mismatch at position −2).

primer in ARMS reactions for both mutations, such that the amplification products of both ARMS tests overlap.

With a multiplex for four or more mutations, a control reaction is not required if mutant and normal ARMS reactions are combined in the same test. In the multiplex, the normal ARMS reactions for ΔF508 and 621 + 1G→T are combined with the mutant over-ARMS reactions for G551D and G542X in the "A"

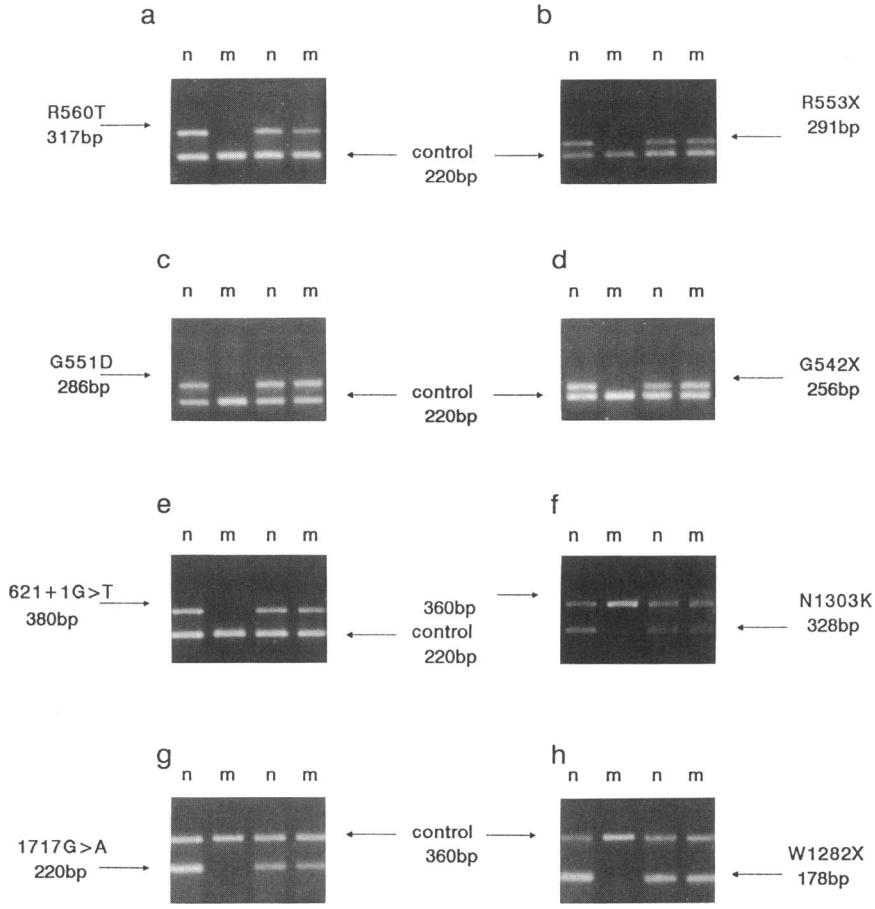

Figure 3 Single ARMS tests. Each panel shows the results obtained using one of the single ARMS tests described in the text. The first two tracks show the results obtained when DNA from a normal individual was used, and the second pair of tracks shows the results of using DNA from an individual heterozygous for the mutation under analysis. The first track in each pair is the product of the normal ARMS reaction, and the second is the product of the mutant ARMS reaction. Each test contains an additional control PCR product, and the location of this band is indicated by an arrow. The ARMS tests are as follows: *a*, R560T; *b*, R553X; *c*, G551D; *d*, G542X; *e*, 621 + 1G→T; *f*, N1303K; *g*, 1717G→A; and *h*, W1282X.

tube, and the corresponding mutant and normal reactions are combined in the "B" tube. Using this format ensures that DNA samples from all possible genotypes should generate at least one PCR product in each tube.

The same methods used to obtain specificity and sensitivity in the single tests were used for the multiplex. In general, altering the primer sequence had a large effect on the yield and specificity of an individual reaction within the multiplex, while small changes were obtained by altering the primer concentrations. The final conditions are summarized in table 4, and typical results of using DNA samples prepared from blood by the rapid method are shown in figure 4.

Validation and Application of the Standard Multiplex Test

The ARMS multiplex was validated by testing 415 samples which had been genotyped using the techniques in table 1. No differences were observed in the genotype derived by either method. A further 216 previously genotyped samples were also analyzed in a blind trial, again with no errors. The reproducibility of the technique was investigated by the repeated testing of nine samples. No variation was seen after 20 repeats. The performance of the test was not affected by a 10-fold increase or decrease in the amount of sample DNA or by altering the annealing temperature ± 2°C.

The multiplex ARMS test has been applied to the following distinct areas:

1. The testing of neonates who have been born with some indication of CF, such as meconium ileus. We have tested 56 such neonates, of whom 11 had meconium ileus. Three were confirmed to have CF (all were homozygous for ΔF508), and two had a single copy of ΔF508, supporting the suspicion of CF. The finding of two mutations provides molecular confirmation of CF.

2. The analysis of couples in which one partner had a relative with CF. A total of 160 such couples have been tested, and a CF mutation found was in both partners in two cases. This resulted in the couple being given a 1-in-4 risk of having a child with CF. In the remainder of couples the risk was reduced to less than 1 in 800 when one partner was definitely a carrier (72 cases) and to less than 1 in 350,000 when both partners were negative.

3. Screening a selective population with no family history of CF. These people are sperm and egg donors and have a prior risk of 1 in 25. If an individual is negative on testing, the risk drops to 1 in 209. A total of 326 sperm or egg donors have been tested, and 11 carriers have been detected; all 11 had the ΔF508 mutation.

4. The testing of 285 individuals with confirmed CF. Both mutations were identified in 77% of cases.

In addition to the validation and application data reported here, the ARMS multiplex is also being tested

Table 4

Sequences and Concentration of ARMS Primers Used in the Multiplex ARMS Test

Mutation and Sequence (5′ to 3′)	Code	Size (bp)	Tube	Concentration (× standard)
ΔF508:				
GACTTCACTTCTAATGATGATTATGGGAGA	DF-C		A/B	1.0
GTATCTATATTCATCATAGGAAACACCACA	DF-j-N	160	A	1.0
GTATCTATATTCATCATAGGAAACACCATT	DF-w-M	157	B	2.0
Exon 11:				
TAAAATTTCAGCAATGTTGTTTTTGACC	11-C		A/B	2.0
G551D:				
GCTAAAGAAATTCTTGCTCGTTACC	GD-j2d3-N	285	B	2.0
AGCTAAAGAAATTCTTGCTCGTTGCT	GD-j-M	286	A	1.0
G542X:				
ACTCAGTGTGATTCCACCTTCTAC	GX-e-N	256	B	1.0
CACTCAGTGTGATTCCACCTTCTCA	GX-M	257	A	1.0
621 + 1G>T:				
TCACATATGGTATGACCCTCTATATAAACT	621-C		A/B	.5
TGCCATGGGGCCTGTGCAAGGAAGTATTCC	621-j-N	380	A	.5
TGCCATGGGGCCTGTGCAAGGAAGTATTGA	621-r-M	380	B	.5

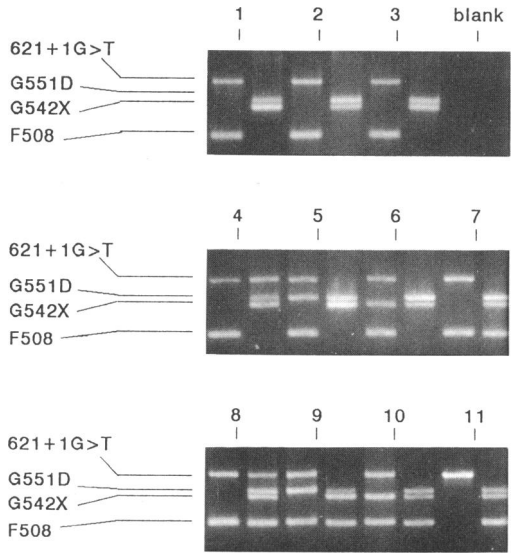

Figure 4 Results obtained using the standard ARMS multiplex test. For each of the 11 samples (lanes 1–11) there are two tracks. The first contains the products of the normal 621 + 1G→T and ΔF508 primers and of the mutant G551D and G542X primers. The corresponding products for the 621 + 1G→T and ΔF508 mutant primers and for the G551D and G542X normal primers are in the second track of the pair. The location of the products of each primer set is indicated at the side of the figure. The genotypes of the 11 samples are as follows: 1, normal; 2, normal; 3, normal; 4, 621 + 1G→T heterozygote; 5, G551D heterozygote; 6, G542X heterozygote; 7, ΔF508 heterozygote; 8, 621 + 1G→T, ΔF508; 9, G551D,ΔF508; 10, G542X,ΔF508; and 11, ΔF508 homozygote.

as part of a large multicenter study. Over 4,000 analyses have now been performed, and the results of these studies will be reported elsewhere.

Discussion

We have demonstrated that the multiplex ARMS technique can be used for the simultaneous detection of mutations of the CFTR gene. During the course of the study, we devised methods for the rapid development of both single and multiplex ARMS tests. A multiplex ARMS test for the four most common CFTR mutations has been developed and tested on over 1,600 samples. The test has been demonstrated to be rapid and reliable, and the resultant diagnoses have been in accordance with confirmatory tests.

Development of Single ARMS Test

The aim of the first part of the study was to develop single ARMS tests specific for individual CF mutations. An ARMS test is specific when (*a*) the yield of product from the target allele exceeds the threshold of detection of the system in use and (*b*) the yield of product from the nontarget allele is not detectable. During development, the PCR reaction conditions were kept the same for all of the ARMS tests, and specificity was achieved by the application of a number of techniques to minimize false priming and maintain a satisfactory level of appropriate priming.

The yield of a PCR product from a particular ARMS primer pair is governed principally by two variables—the rate of hybridization of ARMS primer to the target DNA and the rate at which the bases at the 3′ end of the ARMS primer form a suitable substrate for *Taq* DNA polymerase (Wu et al. 1989). Our data suggest that these are two separate steps, as modification of the 3′ sequence can change the specificity without significantly altering the calculated melting temperature (T_m) (e.g., see fig. 2*d*). Furthermore, the T_m can be increased without altering ARMS primer specificity (e.g., a 40 mer version of the N1303K ARMS primers retained the same specificity; data not shown). As *Taq* polymerase has a clear preference for hybrids which are correctly matched at the 3′ terminus (Kwok et al. 1990), the obligate mismatch due to the mutation of interest was always included at this position. The −2 position of the primers was used for any additional destabilizing mismatches, as this maximized the difference between the rate of substrate formation for the target allele and that for the nontarget allele.

To obtain specificity, with our detection system, most single ARMS reactions required additional destabilization. The choice of mismatched base was determined experimentally, and during the course of the study it became apparent that certain mismatches had a greater destabilizing effect than others. In accordance with Newton et al. (1989), we observed that purine/pyrimidine mismatches were less destabilizing than either purine/purine mismatches or pyrimidine/pyrimidine mismatches (data not shown).

During the development of single ARMS tests, two other approaches were found to be useful in obtaining specificity; these were reducing the primer concentration (e.g., R117H; fig. 2*b*) and inclusion of a control PCR reaction (e.g., R117H; fig. 2*c*). The improved specificity obtained by the inclusion of a control reaction is similar to the effect observed by Sarkar et al. (1990), who improved the specificity of an ARMS test by the reduction of the concentration of dNTP in the reaction. We assume that these approaches work by increasing the competition for reactants in the later linear phase of the reaction (Mullis 1991).

The use of long primers (typically 30 mers) ensured that false priming events were minimized and that primer template interactions were stabilized. Sarkar et al. (1990) report that specificity can be achieved using shorter (less than 20 bp) ARMS primers, but this is associated with artifactual products. The fact that no artifact bands were observed during the course of this study demonstrates the value of using longer primers. A further potential benefit of longer primers is to minimize the disruptive effect of DNA polymorphisms within the target sequence. Our data suggest that the specificity of long ARMS primers is due principally to their 3′ sequence rather than to their T_m. It follows that ARMS will tend to be less affected by internal polymorphisms than are methods relying on T_m for allele discrimination (e.g., ASOs). As we have not yet identified a DNA sample containing such a polymorphism, this benefit has still to be demonstrated experimentally.

Multiplex Test Development

The development of single ARMS tests for any point mutation should be possible using the methods and guidelines described here. It is clear, however, that, for many inherited and acquired mutations, simultaneous analysis of several mutations is desirable. The aim of the second stage of this study was to combine ARMS tests for the most common CF mutations into a multiplex suitable for use in the clinical laboratory.

The multiplex ARMS test was developed using the same PCR conditions that were used for the single tests. With the single tests our primary concern was with the specificity of the ARMS reaction, but with the multiplex tests there was the added complication that the relative yields of the ARMS reactions had to be similar. Initially, the primer sequences selected for the single ARMS tests were combined into multiplex reactions. This approach did not work, because the yields of several of the reactions were too low (G551D-normal, G542X-mutant, and ΔF508 mutant) or too high (621 + 1G→T-normal and mutant) when compared with the yield of the other reactions (data not shown).

A basic equation which describes PCR is $Y_n = (1 + e)^n$, when Y_n is the amplification factor after n cycles at an efficiency e. In a single ARMS test the efficiency is not critical as long as the amount of DNA produced is sufficient to be detected on an agarose gel. In a multiplex, however, there are several competing reactions. If the efficiencies of these reactions are not similar, then each round of the PCR process will amplify the differences in the yields, leading to an imbalance in the system; for example, if the yields of two multiplexed PCR reactions are 0.7 and 0.85, then after 30 cycles there will be a 12-fold difference in the amount of product generated. In practice a balanced multiplex was achieved by modifying the sequence and concentration of the individual ARMS primers.

In general the ARMS primers selected for use in the multiplex had less-destabilizing mismatches than had proved optimal for single ARMS tests. In several cases, primers which had been nonspecific in the single tests were specific in the multiplex, and primers which by themselves were specific gave low product yields. We assume that the efficiency of the PCR using these primers was sufficient to give a detectable product in the single tests but that in the multiplex the competition for reactants in the later stages of the reaction reduced the yield of product to below the threshold of detection.

An additional problem in the development of a multiplex for the four most common CF mutations is the close proximity of G551D and G542X; these mutations are within 28 bp of each other in exon XI of the CFTR gene. A novel approach to ARMS testing, termed "overARMS," was developed to allow the simultaneous detection of these mutations. In this method, ARMS primers are designed for the same template strand, and both use the same common primer. A potential problem with this method is the priming of the G551D product with the G542X primers or product. Other authors have suggested that this approach does not give even yields of product (Main et al. 1991). In our hands the approach worked well and allowed simultaneous detection of G551D and G542X, although the relative yield of these two products was sensitive to changes in mismatch strength and primer concentration.

Validation and Clinical Trial

The ARMS multiplex has proved extremely reliable. During validation the results were in complete accord with those obtained by other methods. After the validation exercise the test was used by two of us (M.J.S. and G.M.) in the Paediatric Genetics Unit of the Royal Manchester Children's Hospital.

The multiplex test made its greatest impact on the speed of delivery of results. On receipt of a sample of whole blood, buccal cells (mouthwash), or chorionic villus biopsy, 88% of CF mutations can be tested within one working day. In the case of neonates suspected of having CF, a rapid result may confirm the

diagnosis long before a sweat test can be performed, and it may be preferable to a sweat test in certain circumstances (Pederenzi et al. 1987). As a comprehensive test for all CF mutations has not yet been developed, any individual who is negative after mutation testing and who is still suspected of having CF must be subjected to a sweat test after a number of weeks.

The test is also useful in the analysis of couples in which one member has a relative with CF. Commonly, such couples are tested when they have a pregnancy underway. The ARMS multiplex is especially useful in this situation, as it allows an accurate assessment of the risk of CF in the fetus to be rapidly obtained.

The testing of sperm and egg donors and recipients occasionally requires rapid results before artificial insemination techniques (AID) are performed. Couples which have a child with CF may avail themselves of the AID facility, and it is essential that sperm donor's carrier risks be minimized.

Finally, since only 1.3% of CF individuals can be expected to be completely negative for all four mutations, the ARMS multiplex may be of some assistance in the diagnosis of a patient. In this situation, any analysis of CFTR mutations is supplementary to—rather than a replacement of—current biochemical diagnostic procedures but can be performed considerably earlier than a sweat test. Further mutation analysis, also performed by the ARMS technique, would normally be carried out in the event of one or both of an individual's CF mutations remaining unidentified.

Conclusions

ARMS is increasingly being used for the genetic analysis of human genomic DNA. Previously, the method has been restricted to the detection of single mutations; here we have demonstrated for the first time the use of multiplex ARMS. Although the development of multiplex ARMS tests is complex, their use is simple and straightforward. The potential application of the method to almost any point mutation or small deletion could lead to the replacement of many of the existing tests used in genetic analysis. Further developments in automation and post-PCR processing could simplify DNA analysis even further.

Acknowledgments

We wish to thank the Cystic Fibrosis Genetic Analysis Consortium for the provision of privileged information. ARMS is the subject of European Patent 0332435, UK Patent Application 9102418.5, and corresponding worldwide patent applications.

References

Ballabio A, Gibbs RA, Caskey CT (1990) PCR test for cystic fibrosis deletion. Nature 343:220

Chehab FF, Doherty M, Cai S, Kan YW, Cooper S, Rubin EM (1987) Detection of sickle cell anaemia and thalassaemias. Nature 329:293–294

Cutting GR, Kasch LM, Rosenstein BJ, Zielenski J, Tsiu L-C, Antonarakis SE, Kazazian HH (1990) A cluster of mutations in the first nucleotide binding fold of the cystic fibrosis conductance regulator protein. Nature 346:366–369

Dean M, White M, Amos J, Gerrard B, Stewart C, Khaw K-T, Leppert M (1990) Multiple mutations in highly conserved residues are found in mildly affected cystic fibrosis patients. Cell 61:863–870

Guillermit H, Fanen P, Ferec C (1990) A 3′ splice site consensus sequence mutation in the cystic fibrosis gene. Hum Genet 85:450–453

Haliassos A, Chomel JC, Tesson L, Baudis M, Kruh J, Kaplan JC, Kitzis A (1989) Modification of enzymatically amplified DNA for the detection of point mutations. Nucleic Acids Res 17:3606

Jeffreys AJ, Macleod A, Tamaki K, Neil DL, Monckton DG (1991) Minisatellite repeat coding as a digital approach to DNA typing. Nature 354:204–209

Kan Y, Dozy A (1978) Antenatal diagnosis of sickle cell anaemia by DNA analysis of amniotic fluid cells. Nature 313:369–374

Kerem B-S, Rommens JM, Buchanan DM, Cox TK, Chakravarti A, Buchwald M, Tsui L-C (1989) Identification of the cystic fibrosis gene: genetic analysis. Science 245: 1073–1080

Kerem B, Zielenski J, Markiewicz D, Bozon D, Gazit E, Yahaf J, Kennedy D, et al (1990) Identification of mutations in regions corresponding to the 2 putative nucleotide (ATP)-binding folds of the cystic fibrosis gene. Proc Natl Acad Sci USA 87:8447–8451

Kobayashi K, Knowles MR, Boucher RC, O'Brien WE, Beaudet AL (1990) Benign missense variations in the cystic fibrosis gene. Am J Hum Genet 47:611–615

Kunkel LM, Smith KD, Boyer SH, Borgaonkar DS, Wachtel SS, Miller OJ, Breg WR, et al (1977) Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. Proc Natl Acad Sci USA 74:1245–1249

Kwok S, Kellogg DE, Spasic D, Goda L, Levenson C, Sninsky JJ (1990) Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res 18:999–1005

Lench N, Stanier P, Williamson RW (1988) Simple non-

invasive method to obtain DNA for gene analysis. Lancet 1:1356–1358

Main BF, Jones PJH, MacGillivray RTA, Banfield DK (1991) Apolipoprotein E genotyping using the polymerase chain reaction and allele-specific oligonucleotide primers. J Lipid Res 32:183–187

Mullis KB (1991) The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. PCR Methods Appl 1:1–4

Myerowitz R (1988) Splice junction mutation in some Ashkenazi Jews with Tay-Sachs disease: evidence against a single defect within this ethnic group. Proc Natl Acad Sci USA 85:3955–3959

Newton CR, Graham A, Heptinstall LE, Powell SJ, Summers C, Kalsheker N, Smith JC, et al (1989) Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res 17:2503–2516

Old JM, Varawalla NY, Weatherall DJ (1990) Rapid detection and prenatal diagnosis of β-thalassemia: studies in Indian and Cypriot populations in the UK. Lancet 336: 834–837

Osborne L, Knight R, Santis G, Hodson M (1991) A mutation in the second nucleotide binding fold of the cystic fibrosis gene. Am J Hum Genet 48:608–612

Pederenzi F, Armani P, Faraguna D, Giglio L, Longo P, Pedrotti D, Zanoni L, et al (1987) Non-CF neonatal hypertypsinaemia. Insights Paediatr 1:19–20

Riordan JR, Rommens JM, Kerem B-s, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1073

Rommens JM, Iannuzzi MC, Kerem B-s, Drumm ML, Melmer G, Dean M, Rozmahel R, et al (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 245:1059–1065

Rommens J, Kerem B-s, Greer W, Chang P, Tsui L-C, Ray P (1990) Rapid nonradioactive detection of the major cystic fibrosis mutation. Am J Hum Genet 46:395–396

Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA (1986) Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific oligonucleotide probes. Nature 324:163–166

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 230:1350–1354

Sarkar G, Cassady J, Bottema CDK, Sommer SS (1990) Characterization of polymerase chain reaction amplification of specific alleles. Anal Biochem 186:64–68

Schwarz M, Summers C, Heptinstall L, Newton C, Markham A, Cain R, Super M (1991) A deletion mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) locus: ΔI507. In: Tsui L-C, Romeo G, Gregor R, Gorini S (eds) The identification of the CF gene: recent progress and new research strategies. Plenum, New York, pp 417–422

Sommer SS, Cassady JD, Sobell JL, Bottema CDK (1989) A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of phenylketonuria. Mayo Clin Proc 64:1361–1372

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Vidaud M, Fanen P, Martin J, Ghanem M, Nicholas S, Goossens M (1990) Three mutations in the CFTR gene in French cystic fibrosis patients: identification by denaturing gradient gel electrophoresis. Hum Genet 85:446–449

Wagner M, Schloesser M, Reiss J (1990) Direct gene diagnosis of cystic fibrosis by allele-specific polymerase chain reaction. Mol Biol Med 7:359–364

Wenham PR, Newton CR, Price WH (1991) Analysis of apoliprotein E genotypes by the amplification refractory mutation system. Clin Chem 37:241–244

Wu DY, Ugozzoli L, Pal BK, Wallace RB (1989) Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757–2760

Wyman A, White R (1980) A highly polymorphic locus in human DNA. Proc Natl Acad Sci USA 77:6754–6758

Zielenski J, Bozon D, Kerem B, Markiewicz D, Durie P, Rommens JM, Tsui L-C (1991) Identification of mutations in exons 1 through 8 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics 10: 229–235