Rapid Cycle Allele-Specific Amplification: Studies with the Cystic Fibrosis ΔF<sub>508</sub> Locus

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Rapid cycle DNA amplification is a polymerase chain reaction technique with improved product specificity and cycle times of 20–60 s, allowing complete 30-cycle reactions in 10–30 min. The presence or absence of the ΔF<sub>508</sub> deletion and wild-type allele was determined in 104 cystic fibrosis patients by rapid cycle DNA amplification. In separate allele-specific assays, sequences on both sides of the ΔF<sub>508</sub> locus were amplified with the 3' end of a discriminating primer at the ΔF<sub>508</sub> locus, with either a 3-bp or a 1-bp mismatch. With rapid cycling (35-s cycles), single-base discrimination was achieved over a broad range of annealing temperatures (50 °C or lower); with conventional cycling and "hot starts" (160-s cycles), only annealing temperatures of 61–62 °C sufficiently discriminated between alleles. With rapid cycling, genotype could still be assessed with annealing temperatures as low as 25 °C. We conclude that faster temperature cycling can improve the results of allele-specific amplification.

Indexing Terms: polymerase chain reaction · heritable disorders · DNA probes · screening

Systematic study of the times required for in vitro DNA amplification reveals interesting facts (1). Thirty cycles can routinely be completed in 15 min or less. Sample denaturation and annealing times of “0”–1 s not only are sufficient, but appear to improve the specificity of reaction. Limitations on the speed of DNA amplification arise not from kinetic requirements of the underlying reactions, but rather from the instrumentation usually used for temperature cycling. The fastest system reported makes use of capillary tubes as containers and air as the heat-transfer medium (1–3). Specificity of reaction is particularly important in allele-specific amplification. Single-base mismatches at the 3’ end of one primer may or may not be amplified, depending on the type of mismatch and reaction conditions (4). The potential advantages of rapid temperature cycling for allele-specific amplification in clinical diagnostics have not previously been tested.

Cystic fibrosis is the most common autosomal recessive disease in Caucasians, with a carrier rate of ~1:25 (5). A 3-bp deletion within the cystic fibrosis transmembrane conductance regulator gene produces a phenylalanine deletion at residue 508 (ΔF<sub>508</sub>) and occurs in 70–75% of cystic fibrosis chromosomes (6). In anticipation of mass screening, much attention has been directed toward simple, reliable, and inexpensive assays for this mutation. Most methods are based on the polymerase chain reaction. Alleles are detected by sequencing (7), allele-specific oligonucleotides (8), restriction enzymes (9), size-fractionation on polyacrylamide gels (10), or allele-specific amplification (11). Allele-specific amplification with direct visualization on agarose gels with ethidium bromide requires the least effort and postamplification processing.

We have applied rapid cycle DNA amplification to the allele-specific amplification of ΔF<sub>508</sub> in 104 patients with cystic fibrosis. For validation, two independent allele-specific amplifications were developed, each for both ΔF<sub>508</sub> and wild-type alleles. The differential amplifications were based on either a 3-bp mismatch or a single base pair mismatch. Temperature cycling conditions for discriminating the single-base mismatch were rigorously determined for both rapid cycling and conventional temperature cycling.

Materials and Methods

Patients with cystic fibrosis were recruited from the Intermountain Cystic Fibrosis Center at the University of Utah. After informed consent, acid/citrate/dextrose-anticoagulated peripheral blood was obtained for purification of genomic DNA. Leukocyte nuclei were digested in 10 g/L sodium dodecyl sulfate and 100–500 mg/L proteinase K at 60 °C for 1 h, extracted with phenol/chloroform, and ethanol-precipitated (12). DNA was resuspended in 10 mmol/L Tris–0.1 mmol/L EDTA by boiling.

DNA amplification reactions were performed with 10 mmol/L Tris, pH 8.3 (at 25 °C), 3 mmol/L MgCl<sub>2</sub>, 50 mmol/L KCl, 500 mg/L bovine serum albumin, 10 g/L Ficoll 400, 1 mmol/L taftazine, and 0.5 μmol of each primer, 200 μmol of each dNTP, 50 ng of human genomic DNA, and 0.4 U of Taq polymerase per 10 μL. One unit (U) of polymerase activity was the amount of enzyme required to incorporate 10 nmol of [3H]dTTP in 30 min at 80 °C, as defined by the manufacturer (Promega, Madison, WI). Mse I was from New England Biolabs (Beverly, MA).

Oligonucleotide primers were synthesized from the phosphoramidites (Gene Assembler Plus; Pharmacia-LKB, Piscataway, NJ). Two amplification primer sets were synthesized, one on each side of the ΔF<sub>508</sub> mutation locus. Primer 1 (GACTTCACTTCTAAAGTCGA) was located at the 5' end of exon 10 of the cystic fibrosis gene (6). The 3’ end of primer 2 (primer 2cf: TACATAGAAGACACCAAT, or primer 2wt: TACATAGAGAAACCCAAA) annealed at the ΔF<sub>508</sub> mutation locus.

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Primers 2cf and 2wt differed from each other only at the 3' base. The other amplification set utilized primer 3 (CTCTTCTAGTTGGCATGCTTT) located at the 3' end of exon 10 and either primer 4cf (CCATAGAAAAATATCATGG) or primer 4wt (CCATAGAAAAATATCATCTTT). Primers 4cf and 4wt differed from each other by three bases at the 3' end.

Temperature cycling equipment included a conventional heat block instrument (DNA Thermal Cycler; Perkin-Elmer Cetus, Norwalk, CT), a second-generation heat block instrument (System 9600; Perkin-Elmer Cetus), and a hot-air cycler (Idaho Technology, Idaho Falls, ID). A custom hot-air cycler was also used for some experiments (1); the temperature/time profiles of the commercial and custom air cyclers were similar. When the conventional heat block instrument was used, all reaction components except the enzyme were mixed (90 μL) and heated to 80 °C. A 10-fold-concentrated enzyme solution at 80 °C was rapidly added and mixed for a "hot start" (13). The reaction mixture was then overlaid with 60 μL of mineral oil at 80 °C. Cycling was performed in "STEP cycle" mode for 35 cycles with denaturation at 94 °C for 35 s, annealing for 35 s at various temperatures, and elongation at 72 °C for 45 s. These times are the minimal denaturation and annealing times necessary for the sample to reach temperature in this system (1). The temperature of a 20-μL sample in a MicroAmp® tube was monitored in the second-generation heat block instrument. Although annealing times of <10 s are "not recommended," we used a 1-s annealing time to minimize the time spent at the annealing temperature. When the air-cycling instrument was used, 10-μL samples were placed in 0.5 mm (i.d.) glass capillary tubes (Idaho Technology). The tubes were used as supplied by the manufacturer without siliconization, and hot starts were not attempted. Thirty-five cycles were performed with denaturation at 94 °C for "0" s, annealing for "0" s at 45 °C unless otherwise specified, and elongation at 72 °C for 10 s. For annealing temperatures of ≤40 °C, the cycler was placed in a cold room at 2 °C.

Amplification products (10 μL) were fractionated by electrophoresis in 1.5% agarose gels unless otherwise stated and made visible by ethidium bromide staining and transillumination with ultraviolet light. The size markers either were a Hae III digest of φ X 174 RF or were produced by cleavage of pUCBM21 DNA with Hpa I and Dra I + HindIII (DNA molecular weight marker VIII; Boehringer Mannheim, Indianapolis, IN). The sample temperature in all systems was monitored with a 0.2 mm (o.d.) thermocouple with a 0.005-s time constant (IT-23; Sensortek, Clifton, NJ), placed in the center of a mock sample.

Results

The expected products were amplified from the cystic fibrosis locus in genomic DNA with rapid cycling (Figure 1). Primer pairs 1 and 2cf/2wt amplify a 147/150-bp product and primer pairs 3 and 4cf/4wt amplify a 80/83-bp product. The identity of the amplification product was confirmed by restriction enzyme digestion; e.g., digestion of the primer 1/primer 2wt product with Mse I gave bands at ~58, 54, and 34 bp (data not shown). The amplifications were allele specific; when there was a mismatch at the 3' end of the discriminating primer, no observable amplification occurred.

The validity of genotype determinations in 104 cystic fibrosis patients was tested by the concordance of the two independent allele-specific amplifications. In the initial testing, performed without knowledge of the results of the other test, 103/104 of the genotypes concurred. The exception was a case that appeared heterozygous with primers 1 and 2cf/2wt, but homozygous ΔF508 with primers 3 and 4cf/4wt. When a sample of the original DNA stock was rediluted, the discrepancy could not be repeated and the patient tested homozygous for ΔF508. The parents were both heterozygous for ΔF508. Sixty of the 104 patients were homozygous ΔF508 (57.7%), 35 were heterozygous (33.6%), and 9 (8.7%) were homozygous wild type. The frequency of the ΔF508 allele was 0.75 in our cystic fibrosis population.

The allele-specific amplification dependent on a single base change (primer 1, primer 2cf/2wt) was optimized for both rapid and conventional temperature cycling. Figure 2 shows amplification products obtained with conventional temperature cycling and hot starts at
Various annealing temperatures. Adequate discrimination occurred with annealing temperatures of 61 and 62°C, but not at 60 or 63°C. Single-base discrimination was lost when the annealing temperature was lowered to 60°C or below. In addition, multiple products of various sizes were amplified with annealing temperatures of 50°C or below. Inclusion of Ficoll and the electrophoresis indicator dye, tartrazine, in the amplification mixture did not affect yield or specificity (data not shown).

Figure 3 shows amplification products obtained with rapid cycling at various annealing temperatures. No product was observed with an annealing temperature of 55°C, a temperature at which very efficient amplification occurred with either matched or mismatched primers on a conventional cycler. Single-base discrimination was achieved with rapid cycling at annealing temperatures of 50°C or lower. Some undesired amplification occurred with annealing temperatures of 35°C or lower. However, most of this undesired amplification resulted in products that were not the size expected for the cystic fibrosis locus, even when the annealing temperature was 25°C (Figure 4).

Figure 5 shows the profiles of sample temperatures near an annealing temperature of 55°C for three different instruments. All systems were empirically adjusted for the fastest possible approach to, and the least possible duration at, 55°C. The sample size used in each instrument was that generally recommended by the manufacturer and varied for each instrument. Samples in the conventional instrument spent about 12 s between 55 and 56°C. The second-generation instrument is capable of faster temperature transitions, especially during cooling, but samples still spend about 8 s between 55 and 56°C. Samples in the rapid cycling instrument are at 55 to 56°C for just under 1 s.

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Fig. 2. Single-base discrimination by DNA amplification in a conventional heat block instrument

Amplification reactions with various annealing temperatures were performed with all four combinations of homozygous normal or homozygous ΔF<sub>508</sub> genomic DNA and normal or ΔF<sub>508</sub> primers. Cycle times varied from 160 s with 62°C annealing to 255 s with 45°C annealing. Hot starts (13) were used with all samples and 35 cycles were performed. Primers were perfectly matched to the template in the samples shown in the top and bottom gel photographs. Amplification of the expected 150-bp (top) or 147-bp (bottom) fragment occurred with annealing temperatures ≤62°C. Because some amplification occurred with the T-T 3′ mismatch at 60°C, only a narrow range (61–62°C) was acceptable for mismatch discrimination. Figs. 2–4. M, molecular weight marker VIII from Boehringer Mannheim

![Diagram](image)

Fig. 3. Single-base discrimination by DNA amplification in a rapid air cycler

Primers and template DNA as described in Fig. 2. Cycle times varied from 30 s at 55°C annealing to 60 s at 25°C annealing because of longer transition times associated with lower annealing temperature. Thirty-five cycles were performed. The optimal annealing temperature for single-base discrimination by rapid cycling differs from that for conventional cycling. With perfectly matched primers, amplification occurred at 50°C but not at 55°C. Single-base discrimination was possible with annealing temperatures of ≤50°C. See Fig. 4 for greater detail
Fig. 4. Allele-specific amplification with 25 °C annealing and rapid cycling
Amplification products were electrophoresed in a 4% agarose gel. Lane 1, ΔF₅₈₀ DNA amplified with ΔF₅₈₀ primers (bottom gel in Figs. 2 and 3). Lane 2, ΔF₅₈₀ DNA amplified with normal primers (A-A mismatch). Lane 3, wild-type DNA amplified with ΔF₅₈₀ primers (T-T 3' mismatch). Lane 4, normal DNA amplified with normal primers (top gel in Figs. 2 and 3). Although some undesired amplification is evident in lanes 2 and 3, most of the products are of different sizes than expected for amplification at the ΔF₅₈₀ locus.

Second-generation instruments can complete 30 cycles in about 1 h (7). "Rapid cycle" DNA amplification, as used here, refers to completing 30 cycles in 10–30 min. Details on the construction of rapid cycling instruments and corresponding sample temperature/time profiles have been published elsewhere (1–3).

Specificity of amplification is particularly important in allele-specific amplification, where detection of an allele may be dependent on fine sequence discrimination, even a single base change. The ΔF₅₈₀ mutation of the cystic fibrosis gene (6) can be detected by allele-specific amplification (11, 12). Several different primer pairs have been devised for discrimination of this mutation from the wild-type allele. Most often, sequences between the ΔF₅₈₀ locus and the 3' end of exon 10 are amplified, and a 3-bp mismatch between the discriminating primers and template is utilized for specificity. The primers used here for 3-bp discrimination (3 and 4cf/4wt) are similar, but not identical, to those previously reported (11).

When sequences between the 5' end of exon 10 and ΔF₅₈₀ are amplified, the bases that can be used to discriminate between ΔF₅₈₀ and wild-type alleles are limited because of the sequence similarity between the ΔF₅₈₀ bases (CTT) and the immediately adjacent 5' sequence (CAT). When a single-base mismatch at the 3' end of the discriminating primers is used (primers 1 and 2cf/2wt), specificity depends on either an A-A mismatch or a T-T mismatch (Figures 2 and 3). A prior attempt to discriminate ΔF₅₈₀ from the wild-type sequence, based on this single-base match/mismatch with use of similar primers, was unsuccessful (14). Under "standard" polymerase chain reaction conditions and with model human immunodeficiency virus templates, a 3' mismatch of T-T did not show discrimination, whereas an A-A mismatch reduced amplification 20-fold (4). In another study, 69 single-base mismatches for allele-specific amplifications included 1 A-A mismatch and 1 T-T mismatch that were reported refractory to amplification (15). Although details are not given, these results were apparently achieved by alteration of primer oligonucleotide and magnesium concentrations. Single-base discrimination from A-A and T-T mismatches have only rarely been reported and seemed good tests for the putative specificity advantages of rapid cycling.

The redundancy of using two allele-specific amplifications for ΔF₅₈₀ can be used to detect infrequent errors. With rapid temperature cycling for ΔF₅₈₀ genotype determination, 1 of 104 paired analyses was initially discordant. Repeat analysis of the discrepancy showed that the error occurred with the primer 1–2cf/2wt amplification, dependent on single-base discrimination. Use of two separate allele-specific amplifications can prevent rare technical errors, albeit at twice the work. Genotyping errors have also been reported with the polymerase chain reaction because of unexpected polymorphisms at primer binding sites (16); these would also be detected in a redundant system.

A total of ~3 h was required for the DNA purification from blood, amplification, and electrophoresis described.
here. The DNA purification was the most time-consuming step (2 h). Although the genotyping system described here is rapid and inexpensive, it is not ideal for population screening. Current capillary-based systems are awkward when large numbers of samples are manipulated. Automated systems that detect not only ΔF_{508} but also multiple cystic fibrosis mutations (for a 90% detection rate) are desirable for population screening (17).

Nevertheless, an understanding of the factors contributing to "nonspecific" amplification is important not only for ΔF_{508} testing, but also for any allele-specific amplification and for polymerase chain reaction techniques in general. Single-base discrimination with conventional cycling and hot starts (13, 18) was possible only in a narrow annealing range of 61–62°C (Figure 2). At 60°C, the T-T mismatch failed to prevent amplification. At 55°C, both the T-T and A-A mismatches were extended with good efficiency, leading to complete failure of the test for genotype.

With rapid cycling, optimal annealing temperatures for single-base discrimination were about 15°C lower than for conventional cycling (Figure 3). Whereas conventional cycling required a precise annealing temperature of 61–62°C, a wide range of annealing temperatures at ≤60°C was acceptable with rapid cycling. Although the claimed precision of most instruments is better than 1°C, temperature variations greater than this have been reported (19). A wide range of acceptable annealing temperatures increases tolerance for calibration errors, minor compositional differences between samples, and sample-to-sample temperature variations. Surprisingly, any annealing temperature between 25°C to 50°C could be used to genotype with rapid cycling. Even with annealing at 25°C, the major undesired amplification products from rapid cycling were of a different size from those expected for the cystic fibrosis locus (Figure 4). In contrast, single-base specificity was entirely lost at an annealing temperature of 55°C with conventional cycling, even with hot starts (Figure 2).

It is interesting that the optimal annealing temperatures for conventional and rapid cycling are so disparate. The shape of the sample temperature/time curve near the annealing temperature is very different for rapid and conventional cycling (Figure 5). Both the temperature transition rates and the minimum amount of time that can be spent at the annealing temperature vary greatly. Sample annealing times of <1 s can be achieved with rapid cycling; the minimal annealing times in conventional cycling are 10 times longer, even with second-generation instrumentation. The short annealing times and (or) the rapid temperature transitions must be important for the single-base specificity of rapid cycling.

Nonspecific amplification in the polymerase chain reaction usually refers to either template-independent amplification (primer dimers or oligomers) or amplification of template regions other than the desired target. Amplification of such nontarget sequences generally leads to products of sizes different from the desired sequence. Hot starts have been used to minimize this kind of undesired amplification and allow single-copy detection after amplification with ethidium bromide staining (18). Nevertheless, hot starts will not prevent undesired amplification if the annealing temperature during cycling is too low (Figure 2).

With allele-specific amplification, an additional kind of nonspecific amplification can occur. A mismatched primer may anneal at the expected sequence but extend through the mismatch, giving a product of the same size as a perfectly matched primer. This kind of undesired amplification means failure of the method as a test for genotype. Rapid cycling strongly disfavors extension of a mismatched primer annealed at the expected site and should be useful in the design and implementation of other allele-specific amplifications.

A detailed study of the required annealing times for different annealing temperatures in DNA amplification and their effect on product yield and specificity has not been performed. A rigorous analysis based on annealing kinetics and polymerase extension rates is also conceivable. However, such studies are limited by current instrumentation. Conventional temperature cyclers have long transition times, whereas current rapid cycling instruments lack full control of temperature transition rates.

We gratefully acknowledge Idaho Technology for providing a commercial hot-air rapid cycler and Associated Regional University Pathologists, Inc., for financial assistance. The University of Utah has applied for a patent on rapid air cycling instrumentation. This technology has been licensed to Idaho Technology, in which C.T.W. holds equity interest.

References
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