The recent discovery of the cystic fibrosis gene enables DNA-based testing for the direct identification of the deletion of three basepairs coding for phenylalanine at codon 508, the major mutation responsible for the disease. This mutation can be detected by analysis of amplified DNA with allele-specific oligonucleotide probes. We have simplified the procedure of Kerem et al. (Science 1989;245:1073–80) so that the assay can be routinely completed in one working day, starting with an extracted DNA sample. Addition of salmon-sperm DNA to the product of the polymerase chain reaction greatly improved the quality of the hybridization signal. The precision of the method was evaluated by blind analysis and interpretation of results for 100 specimens from 25 patients. The same result was obtained for each patient analyzed separately four times, and four independent observers agreed on the interpretation of results for all 100 specimens. No specimen required repeat analysis to produce interpretable results. We conclude that this method is reliable and convenient for routine clinical laboratory use.

Additional Keyphrases: screening • heritable disorders • gene probes • polymerase chain reaction

The cystic fibrosis (CF) gene has recently been isolated and the major mutation responsible for CF has been identified as a 3-basepair (bp) deletion at codon 508, which deletes a phenylalanine residue (ΔF508) (1, 2). This mutation, present in ~70% of CF chromosomes (3), can be detected directly by DNA amplification by the polymerase chain reaction (PCR), followed by electrophoresis, Southern blotting, and analysis with normal and ΔF508 allele-specific oligonucleotide (ASO) probes (3). This discovery will have a major impact on clinical testing because of the high prevalence of CF carriers in the Caucasian population.

Although population screening for this mutation is not yet recommended practice (4), it is important to develop and evaluate methods suitable for potential high-volume testing. Evaluation of assay reliability and quality control have also been identified as critical issues in implementation of CF screening programs (4). In addition to the PCR–ASO method, several electrophoretic methods have been reported for detecting the ΔF508 mutation (5–8). These methods may be advantageous in certain laboratory settings, but there has been less clinical experience with them. Moreover, electrophoretic methods may not be the most desirable for large-scale testing programs because of the limitations on the number of samples that can be analyzed at one time and the difficulty in automating these methods.

We have therefore developed a simpler and more rapid version of the PCR–ASO test (9). The method shortens the PCR step and eliminates electrophoresis; other steps have also been shortened to allow the procedure to be completed in one day. We have also evaluated the performance of this method in a blind study of 100 specimens, and also in routine clinical use.

Materials and Methods

Genomic DNA was prepared from 5 mL of EDTA-anticoagulated whole-blood samples by standard methods (9). DNA was dissolved in 1000 μL of a buffer composed of, per liter, 10 mmol of Tris·HCl, 10 mmol of NaCl, and 1 mmol of EDTA (pH 7.6). DNA concentrations spectrophotometrically determined to exceed 200 mg/L were diluted to ~100 mg/L.

DNA was amplified by PCR, with use of a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). The oligonucleotide primer sequences amplify a 98-bp fragment that includes codon 508 of the normal CF gene (3). Each 50-μL PCR reaction mixture consisted of 5 μL (0.2–1.0 μg) of the prepared DNA sample, 50 pmol of each primer, 1.25 U of Taq polymerase (AmpliTaq: Perkin-Elmer Cetus, Emeryville, CA), each dNTP at 200 μmol/L, MgCl₂ at 1.5 mmol/L, Tris·HCl (10 mmol/L, pH 8.3), KCl at 50 mmol/L, gelatin at 0.1 g/L, and one drop of mineral oil. All reagents (except the genomic DNA specimen and mineral oil) could be mixed in advance and stored at ~20 °C in single-use aliquots. PCR amplification conditions were as follows: (a) 6 min at 94 °C; (b) 30 cycles consisting of 1 min at 94 °C and 1 min at 62 °C. Oligonucleotide probes (15 pmol) for detection of the normal DNA sequence and the ΔF508 mutation (3) were 5'-end-labeled with [γ-32P]ATP (8 pmol, 6000 kCi/mol; DuPont NEN Research Products, Wilmington, DE) by standard methods (10) to a specific activity of 4×10¹⁰ dpm/mol.

We denatured 10 μL of the PCR reaction product with 1.0 μL of 3.5 mol/L NaOH reagent containing salmon-sperm DNA (Sigma Chemical Co., St. Louis, MO), 1 g/L, and let this stand at room temperature for 15 min. We then applied 3.3 μL of this mixture to a dry nylon membrane (Genescreen-Plus; DuPont NEN) and air-dried it at room temperature for 30 min or at 37 °C for 15 min. A negative control (a PCR reaction with no added DNA) was also applied to each membrane. Duplicate membranes were prepared for hybridization with the normal and ΔF508 probes. Membranes were placed in a prehybridization solution (per liter, 0.9 mol of NaCl, 0.2 mol of Tris (pH 8.0), 5 mmol of EDTA, 5 g of non-fat powdered milk, and 10 g of sodium dodecyl sulfate) at 37 °C for 10 min, then hybridized at 37 °C for 1–2 h in a solution consisting of the prehybridization solution plus the radiolabeled probe, 1.5×10⁶ dpm/L.

The membranes were first washed for no more than 10 min at room temperature in a solution of (per liter) 15 mmol of sodium chloride, 1.5 mmol of sodium citrate, and 10 g of sodium dodecyl sulfate. Membranes were washed a
second time in the same solution at 37 °C for exactly 1 min. We evaluated the membranes by autoradiography, exposing the membranes to roentgenographic film (XAR-2; Eastman Kodak, Rochester, NY) at −70 °C with an intensifying screen for 30 min to 2 h.

In a blind study devised to evaluate the reproducibility of the PCR–ASO method, we divided coded DNA specimens from 25 individuals into four aliquots, making 100 total samples. These samples came from normal individuals, chosen without conscious bias, as well as CF patients and carriers. We assayed these samples in no set order, using the PCR–ASO dot-blot method on seven different days over four weeks. Results were interpreted independently by four individuals. One individual then estimated the intensity of the signal from each sample on an integer scale from zero to three, with zero being the background signal of the negative control.

We also used the method to determine ΔF508 status in samples from 39 patients. The rationale for clinical testing of these patients varied: suspected CF, family history of CF, or spouse of a CF carrier. These specimens were evaluated over four months and interpreted separately by at least three individuals.

**Results and Discussion**

Typical results of the PCR–ASO dot-blot method for ΔF508 detection are shown in Figure 1A. We found that adding salmon-sperm DNA to the PCR product dramatically increased the intensity and uniformity of the hybridization signal (cf. Figure 1A and B). This apparent carrier effect was also observed when at least 3 μg of unamplified human genomic DNA was added to the PCR product before application to the membrane. A simple dot-blot gave good discrimination between normal and mutant alleles, indicating that the electrophoresis and Southern blotting steps of the original procedure (3) can be omitted. Investigation of the amplification conditions showed that the shortened PCR profile, omitting the usual extension step, was sufficient to amplify the desired product. No vacuum manifold was necessary; placing the PCR product directly on the dry membrane gave excellent results. Washing the membranes for 1 min at 37 °C efficiently discriminated between the normal and the mutant alleles, probably because of the three-base difference in DNA sequence. Other simplifications were also introduced to shorten the procedure (see Materials and Methods). The entire procedure, from PCR set-up to interpretation of autoradiograms, takes about 6–8 h of elapsed time.

Our experience showed that prepared hybridization solutions containing labeled probes could be used and reused repeatedly if the solution was frozen at −20 °C between uses. Hybridization solution handled in this way was used successfully as many as six times over a four-week period. Thus, routine use of this method does not require frequent probe labeling. The estimated reagent cost for labeling two ASO probes, based on a six-time usage schedule, was $5.00 per use. This represents the maximum probe cost; each probe probably can be used even more frequently because it is added in large excess.

The intensity of the dot-blot signal was independent of the amount of genomic DNA added to the PCR reaction in the range from 0.05 to 5.0 μg. Thus, because the amount of DNA added to each PCR reaction is not critical, quantification of DNA samples is not necessary. The elimination of this step further simplifies the procedure.

In the blind evaluation of the PCR–ASO method, there was complete agreement between the four individuals who interpreted the autoradiograms. Further, none of the 100 samples required repeat analysis for interpretation. The interpretation of the results for each individual indicated that nine were heterozygous carriers of the ΔF508 deletion, 14 showed hybridization only with the normal probe, and two showed hybridization only with the ΔF508 probe. All results were consistent with the clinical history of each patient.

The ΔF508 results for the 39 other clinical samples showed that 23 samples hybridized only with the normal probe, 12 were from heterozygous carriers of the ΔF508 mutation, and four hybridized only with the ΔF508 probe. Each sample gave sufficient signal for interpretation, so no repeat analyses were required. There was unanimous agreement between individuals interpreting the autoradiograms.

To further evaluate the precision of the method, we scored the intensity of the signal produced by each blind study sample. These results indicate that the between-assay variability in scored signal intensity of each sample was no more than ± one integer. For the range of DNA added to each PCR reaction (0.2–1.0 μg), the sample score was found not to be related to the total amount of added DNA; however, the score was related to the number of alleles with the normal and ΔF508 sequence. Specimens showing an observable signal with only one probe had an overall mean score of 2.76 (SD 0.23, n=16). The overall mean score for specimens showing hybridization with the normal and ΔF508 probes was 2.05 (SD 0.24, n=18). The difference between the two means was statistically significant, t=8.37 (t_{0.05}=1.76).

Several electrophoretic methods for detecting the ΔF508 mutation have recently been described. One promising method relies on the formation of a DNA heteroduplex (5),

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**Fig. 1.** Representative autoradiogram (1-h exposure) of controls and 16 patients' samples analyzed by the PCR–ASO dot-blot method. Before application to the membrane, identical PCR products (3 μL per dot) were diluted in the presence (A) and absence (B) of salmon-sperm DNA (90 ng/μL, final concentration) as described in the text. Samples are labeled by row and column (r,c). Specimens appearing in (1,1), (1,2), and (1,3) are controls from normal, carrier, and affected CF individuals, respectively. The negative PCR control (4,5) shows no signal.
which forms under appropriate conditions when the ΔF508
and normal alleles are present. The heteroduplex migrates
more slowly than do homoduplex fragments and can be
separated in 20 min by electrophoresis and detected with
ethidium bromide staining. To reliably distinguish ho-
mozygous normal and affected individuals with this
method, two electrophoresis lanes per sample must be run.
One lane contains the PCR-amplified patient’s sample, and
the other contains the same PCR product supplemented
with an equivalent amount of PCR product from a noncar-
ier of the mutation (5). Alternatively, the ΔF508 mutation
has been detected directly by 2-h electrophoresis followed
by ethidium bromide staining (6) or autoradiography from
emission of a radiolabeled primer used for PCR amplifica-
tion (7). An overnight restriction enzyme digestion of PCR
product followed by a 2-h electrophoresis has also been a
successful method of detecting the ΔF508 mutation (8).

Electrophoretic methods will probably be most suitable
for laboratories with a low volume of testing. Although
these methods do not require radiolabeled probes, they do
require the reagents, labor, and equipment associated with
electrophoresis. Comparison of methods should take into
account time associated with gel preparation, loading, and
staining, as well as the number of specimens that can be
evaluated, given the size of the electrophoresis apparatus.
Unlike the ASO–PCR dot–blot method, which has the
potential for the automation of membrane preparation and
data analysis, these tasks must be done manually for
electrophoresis. Additionally, possible analytical problems
associated with sample overload, variation in migration,
nonspecific bands, and differences in the amount of PCR
product have not been ascertained for electrophoresis.

The ASO–PCR dot–blot method described will probably
be most appropriate for higher-volume testing programs,
such as those for newborn or population carrier screening.
With this method, one can hybridize, wash, and analyze
membranes containing hundreds of specimens at one time.
Compared with electrophoresis, this method can provide
higher sample throughput, amenability to automation, and
the demonstrated low incidence of repeat analysis. Adapt-
ing the method to tolerate impure DNA samples, thereby
minimizing sample preparation time, would further in-
crease its utility for clinical testing and screening.

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Determination of β-Carotene and Its Cis Isomers in Serum

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All-trans-β-carotene was resolved from its cis isomers in
human serum by reversed-phase “high-performance” liquid
chromatography. Absorption spectra of the cis peak sug-
gested that 13-cis-β-carotene was the predominant cis iso-
er. Analyses and recovery studies of fresh and stored sera
eliminated the possibility that isomerization had occurred in
samples during handling or storage. The average analytical
recovery was 101.9% for standards of the all-trans, 9-cis,
and 13-cis-β-carotene compounds in pooled serum samples.
We also demonstrated that cis isomers that had not formed
after the blood was drawn and that cis isomers of β-carotene are
present at significant concentrations in the human circulation.

Additional Keyphrases: chromatography, reversed-phase
nutritional status

All-trans-β-carotene (ATBC) in fruits and vegetables has
been shown to undergo cis isomerization during processing
(1–7), with the 9-cis-β-carotene (9-CBC) and 13-cis-β-carot-
ene (13-CBC) forms being the predominant isomerization
products.2 Thus consumption of a typical diet involves
ingestion of significant quantities of cis isomers. However,
the metabolic fate of the isomers is unknown. Several
animal-feeding studies, measuring growth or the concen-
tration of vitamin A in tissue after ingestion of β-carotene
isomers, have demonstrated that cis rotation of ATBC can
substantially reduce its provitamin A activity (8–11).

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2 Nonstandard abbreviations: ATBC, All-trans-β-carotene;
9-CBC, 9-cis-β-carotene; 13-CBC, 13-cis-β-carotene; and NMR,
nuclear magnetic resonance.

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