
DNA Diagnosis with Mutation-Specific Artificial Methylation Sites: Application to Rapid Screening of ΔF508

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Many polymerase chain reaction (PCR)-based methods for diagnosis of minute mutations are suboptimal for automated screening because of their reliance on gel electrophoresis or probe hybridization. In the method reported here, PCR products containing artificial methylation sites are analyzed by measuring incorporation of radiolabeled methyl groups. Primers are designed to amplify the possible mutation-containing region such that the 3' end of one primer lies adjacent to the possible mutation. Sequence modification near this end creates either a mutation- or wild type (WT)-specific artificial methylation site in the PCR product. The product is briefly incubated with an appropriate DNA methylase and tritiated S-adenosylmethionine ([3H]SAM), separated from free SAM by column chromatography, and analyzed for incorporation of tritium. Applying this technique to the cystic fibrosis ΔF508 deletion, we accurately diagnosed five homozygotes, five heterozygotes, and five normal individuals within 40 min of PCR completion. The method can be generalized to rapid, automated detection of a variety of point mutations and small deletions.

Additional Keyphrases: polymerase chain reaction · cystic fibrosis · heritable disorders

The polymerase chain reaction (PCR) for in vitro DNA amplification has revolutionized the diagnosis of disease by DNA analysis (1, 2).1 Diseases caused by large deletions (e.g., Duchenne muscular dystrophy) or point mutations within a restriction site (e.g., sickle cell disease) can be readily diagnosed by PCR amplification and gel electrophoresis—in the latter case, after restriction enzyme digestion (3, 4). Single base changes or minute deletions not altering a restriction site have been detected by hybridization of PCR products to allele-specific oligonucleotides (5–7), allele-specific PCR (8–11), and PCR-based introduction of artificial, mutation-specific restriction endonuclease sites (12, 13). Recently, Jalkanco et al. (14) reported the use of solid-phase minisequencing for detection of defined cystic fibrosis (CF) mutations. This method offers two advantages: It gives quantitative results and lends itself to automation.

The method reported here (Figure 1) also provides rapid detection of known minute mutations in amplified DNA fragments, without gel electrophoresis, by introducing mutation and wild type (WT)-specific artificial methylation sites during PCR. Analysis of the product involves incubation with the appropriate DNA methylase and tritiated S-adenosylmethionine ([3H]SAM), and

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1 Nonstandard abbreviations: CF, cystic fibrosis; MUT, mutant; PCR, polymerase chain reaction; SAM, S-adenosylmethionine; WT, wild type; and HET, heterozygous.

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Fig. 1. Schematic representation of DNA diagnosis of ΔF508 by use of artificial methylation sites
MUT, mutant: HET, heterozygous; WT, wild type

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assay of incorporated tritium. This process is inexpensive, gives numerical results in <40 min after completion of PCR, and could be automated.

To assess the diagnostic value of this method, we analyzed DNA samples for the ΔF508 deletion found on 70–75% of chromosomes carrying the CF gene from North American patients with CF (15, 16).

Materials and Methods
DNA Samples
We extracted DNA from peripheral blood leukocytes of individuals with CF and their first-degree relatives by lysis with sarcosyl, digestion with proteinase K, and extraction with guanidine hydrochloride (17). DNA samples were subsequently analyzed for the presence or absence of the ΔF508 3-base-pair (bp) deletion by one or both of the following methods. In the first, we amplified DNA by PCR, using primers immediately flanking the deletion. The resulting 54-bp mutant (MUT) vs 57-bp WT products were separated by electrophoresis on a 5% NuSieve agarose gel (FMC Bioproducts, Rockland, ME). In the other method, we distinguished alleles with the ΔF508 deletion (MUT) from WT alleles by allele-specific PCR (18). In the second method, amplification with one primer pair resulted in a product only if the deletion was present; amplification with a second primer pair resulted in a product only if the WT sequence was present. We used five homozygous MUT, five homozygous WT, and five heterozygous (HET) genomic DNA samples in this study.

PCR Primer Design
We designed two pairs of primers to introduce artificial methylation sites (Figure 2). CF Cla I (anti-sense: TAT TCA TAG GAA ACA TCG A) and CF5PR (GAC TTC ACT TCT AAT GAT G) produced a 150-bp fragment containing a Cla I methylase site if the template DNA had the ΔF508 deletion. CF dam (GCA CCA TTA AAG AAA ATA TGA T), whose 3’ end directly flanked the ΔF508 site and CF5PR (anti-sense: TTC TAG TTG GGA TGC TTT), produced an 80-bp product that contained a dam site only when WT DNA was amplified.

PCR Protocol
Each 100-μL reaction mixture contained 0.5–1.0 μg of genomic DNA, 0.3 U of Taq polymerase (Promega, Madison, WI), 1 μmol/L of each primer, 0.5 mmol/L spermidine, 1.5 mmol/L magnesium, 200 μmol/L for each dNTP (dATP, dTTP, dCTP, dGTP), and 1× PCR buffer (Promega supplies this as 10×: 500 mL of glycerol, 50 mmol of Tris-HCl, pH 8.0, 100 mmol of NaCl, 0.1 mmol of EDTA, 1 mmol of dithiothreitol, and 10 mL of Triton X-100 per liter). Samples underwent 35 cycles of PCR (95 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min) with a final extension at 72 °C for 5 min.

Restriction Digestion
We incubated 20-μL aliquots of PCR products amplified with the MUT-specific primers for 2 h at 37 °C with 10 U of Cla I restriction endonuclease before and after treatment with Cla I methylase (New England Biolabs, Beverly, MA). Twenty-microliter aliquots of PCR products amplified with the WT-specific primers were incubated for 2 h at 37 °C with 20 U of Mbo I restriction endonuclease before and after treatment with dam methylase (New England Biolabs). We analyzed digests by agarose gel electrophoresis (3% GTG (NuSieve) and 1% LE agarose mixture (SeaKem; FMC BioProducts)).

Methylation Reaction with [3H]SAM and Analysis of Products
We added 10 U of Cla I methylase to each 20-μL aliquot of the PCR products from the MUT-specific primer pair, and 16 U of dam methylase to each 20-μL aliquot of PCR products from the WT-specific primer pair. We added labeled SAM (1.5 μCi of [3H]SAM, specific activity 85 kCi/mol; Amersham International, Bucks, UK) to every sample and allowed the resulting reaction mixtures to incubate for 30 min at 37 °C.

We separated PCR products from unincorporated [3H]SAM on separate NICK columns (Sephadex G-50 gravity columns; Pharmacia, Piscataway, NJ). The MUT-specific fragments were eluted from their column with 600 μL of a solution of Tris (10 mmol/L) and EDTA (1 mmol/L), and the WT-specific fragments were eluted from their column with 550 μL of this solution. We placed the eluate from each sample directly into 2 mL of scintillation fluid (Ecocint; National Diagnostics, Mansville, NJ) for direct assay of 3H.

Results
The creation of MUT-specific Cla I methylation sites and WT-specific dam methylation sites by PCR-mediated base substitution was verified by restriction endonuclease digestion (Figure 3). DNA homozygous for the ΔF508 deletion and amplified with the MUT-specific primer pair was completely digested with Cla I restriction endonuclease, yielding 131- and 19-bp digestion fragments.
products, whereas WT DNA amplified with the MUT-specific primer pair was not cut by Cla I. Heterozygous DNA generated both cut (131-bp) and uncut (150-bp) fragments. Digestion with Mbo I (same recognition sequences as dam methylase) of PCR products amplified with the WT-specific primer pair showed the presence of a dam site only in WT and HET but not in MUT DNA samples.

When we amplified genomic DNA with MUT-specific primers and treated it with Cla I methylase in the presence of $[^3]H$SAM, MUT DNA gave a five-sample average of 16,000 disintegrations per minute (dpm) (range: 10,000–25,000 dpm), WT DNA averaged 2200 dpm (900–3600 dpm), and HET DNA averaged 4000 dpm (1700–6500 dpm). With the WT-specific primers and methylation with dam, the average dpm was 10,300 for WT samples (4000–23,000 dpm), 1400 dpm for HET samples (1000–2000 dpm), and 700 dpm for MUT samples (300–960 dpm) (Figure 4). Table 1 shows the calculated ratio of MUT-specific to WT-specific disintegrations per minute for each sample.

**Discussion**

The method presented here accurately classified 15 individuals as WT, HET, or MUT with respect to the ΔF508 mutation. As predicted, homozygous mutants gave high counts with MUT-specific primers and Cla I methylase, and homozygous WT individuals gave high counts with WT-specific primers and dam; heterozygotes gave intermediate counts with both the MUT- and WT-specific systems. The finding that HET samples gave less than half as many disintegrations per minute as MUT samples methylated with Cla I methylase or as WT samples methylated with dam methylase may reflect the fact that PCR products from heterozygotes could contain 50% heterodimers, which are not substrates for methylases. The incorporation of $[^3]H$ would then be one-fourth that seen in homozygotes, approximately the observed result.

Concurrent use of MUT-specific primers with Cla I methylase and WT-specific primers with dam methylase enables clear distinction among HET, WT, and MUT samples. The calculated ratio of MUT- to WT-specific disintegrations per minute yielded unambiguous results: ratios >10 were seen in all MUT, <1 were seen in all WT, and >1 but <5 were seen in HET DNA samples (Table 1). These ratios could be computer-generated from scintillation counters for immediate
printout of numerical results. Ideally, a screening test should have a very high positive predictive value (i.e., the false-positive rate should be low) and, more important, a very high negative predictive value (i.e., no carriers should be missed). The results of this small study suggest that use of the WT-specific primers with dam methylase alone would yield adequate test characteristics, because there was no overlap in the range of incorporation of $^{3}H$ into HET and WT samples. Analysis of additional samples is required to firmly establish the lack of overlap between HET and WT samples before this method is applied in a clinical setting. The overlap between HET and MUT is not as important for carrier screening because unrecognized $\Delta F_{508}$ homozygotes are rare and, in any case, a positive test result would require further study that would give a definitive diagnosis.

A theoretical source of false-positive and false-negative results would be mutations adjacent to the site of the disease-causing mutation, which might lead to alterations of the desired artificial methylation site. The known missense mutations in the $\Delta F_{508}$ region (19) would not abolish the artificial Cla I or dam sites used in our method. The disease-causing $\Delta I_{507}$ mutation, which lies adjacent to the F508 codon (20), would give a result different from WT or $\Delta F_{508}$ alleles. With use of the primers CF Cla I and CF5PR, a WT product would be generated that would not be a substrate for Cla I methylase. With use of CF dam and CF5PR, chromosomes bearing $\Delta I_{507}$ would yield no product because the region of genomic DNA corresponding to the last three bases of the CF dam primer would be deleted. The resulting sequence would contain three consecutive mismatches for the 3' end of the primer. Homozygotes for $\Delta I_{507}$ would generate low incorporation in both WT- and MUT-specific assays. $\Delta I_{507}$/WT heterozygotes would yield intermediate counts with WT-specific and low counts with MUT-specific primers. $I_{507}$/F508 compound heterozygotes would give low counts with the WT-specific and intermediate counts with the MUT-specific reaction.

This method could be generalized for detection of other known mutations that alter naturally occurring methylation sites or mutations for which PCR primers can be designed to introduce MUT- and WT-specific methylation sites. For many restriction enzymes, a corresponding methylase has been isolated that recognizes the same site. At least 13 different sites can be detected with currently available methylases, allowing for application of this method to most mutations. Commercial development of additional methylases would further extend the applicability of this technique.

The advantages of this method include the speed with which numerical results are obtained and the feasibility of automating nearly the entire procedure. As in all diagnostic testing that incorporates PCR technology, minute amounts of sample can be used as starting material. One disadvantage of this method is its reliance on the use of a radioactive label. However, $^{3}H$ is a very weak beta-emitter, and the radiation hazard related to its use is minimal.

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References


