Detection of Cystic Fibrosis ΔF508 Mutation by Anti-Double-Stranded DNA Antibody*†

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ABSTRACT

This study evaluated an enzyme immunoassay (EIA) as a screening tool for detection of the most common mutation (ΔF508) in cystic fibrosis (CF). Guthrie card bloodspots were extracted to remove whole blood polymerase chain reaction (PCR) inhibitors. The washed filter paper was directly amplified under standard (98 bp amplicons) or modified PCR conditions (491 bp amplicons) for the ΔF508 mutation. Monoclonal anti-double stranded deoxyribonucleic acid antibody was used to detect competent hybrid formation between PCR products and normal (N) and mutant (M) cDNA (deoxyribonucleic acid) probes coated to microtiter plate wells. Under standard conditions, mean relative color production (N/M) via an enzyme-linked horseradish peroxidase secondary antibody was 8.8, 0.6 and 0.04 for individuals normal, heterozygous and homozygous for the CF ΔF508 mutation, respectively (n = 27). Comparison of EIA results to those obtained by tris-borate-EDTA/8 percent polyacrylamide gel electrophoresis (TBE-PAGE) yielded excellent correlation (100 percent) for all three genotypes (n = 27). In comparison to TBE-PAGE, the EIA was 5 to 10 fold more sensitive when serially diluted PCR samples were evaluated. Larger PCR products (491 bp amplicons) for the CF ΔF508 mutation obtained under modified conditions were not resolved by TBE-PAGE. The EIA, however, demonstrated equal sensitivity to the 98 bp and 491 bp amplicons. Performance time for TBE-PAGE analysis was substantially shorter (25 percent) than the EIA (3.5 to 4 h and 4.5 to 5 h, respectively) when small batches of samples (n = 5) were analyzed. The TBE-PAGE was not, however, convenient for screening large numbers of PCR-amplified samples (n > 15).

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Introduction

The ΔF508 mutation, the most common genetic defect in individuals with cystic fibrosis (CF) (approximately 70 percent of all cases), results in a three base pair (bp) deletion in the CF transmembrane conductance regulator gene. Detection of amplified polymerase chain reaction (PCR) products for the ΔF508 mutation has been accomplished by agarose and polyacrylamide gel electrophoresis in tris-borate-ethylenediamine tetraacetic acid (TBE-PAGE) with ethidium bromide staining, allele specific oligonucleotide (ASO) hybridization (e.g., Southern, dot blots), and restriction endonuclease mapping. These qualitative detection techniques are, however, subjective, time consuming, labor intensive, and, thus, not generally applicable for screening or routine laboratory purposes.

Because of their high specificity and potential for automation, ASO hybridization adapted to microtiter plate format provides an excellent alternative to more complicated detection methods. To facilitate routine laboratory use, a number of investigators have labelled deoxyribonucleotides, oligonucleotide primers, and cDNA probes with nonradioactive ligands including biotin, digoxigenin, fluorescent compounds, rare-earth lanthanide chelates (Eu, Sm, Rb), as well as, enzymes. Although these happenings can be easily detected by a variety of colorimetric and fluorescent methods, modification of PCR products and cDNA probes may alter hybridization efficiency. To avoid the introduction of modifying groups into nucleic acid sequences, a monoclonal ant double stranded deoxyribonucleic acid antibody (anti-dsDNA) was developed and adapted for enzyme immunoassay (EIA). The anti-dsDNA was subsequently shown to be highly specific for double-stranded versus single-stranded DNA. Thus, the antibody provided an ideal and widely applicable tool for specific detection of competent ASO hybridizations irrespective of nucleic acid sequence. Later research studies demonstrated that the anti-dsDNA EIA was useful for HLA typing, detection of infectious disease (HIV, HBV), as well as, genetic defects (CF ΔF508 mutation).

As part of our neonatal screening program for CF, a simple and sensitive method was developed for the direct amplification of Guthrie card bloodspot DNA. Previous work, however, with the anti-dsDNA EIA indicated that target copy number was a critical factor in assay sensitivity. Because the limited amount of genomic material entrapped in the filter paper matrix (approximately 150 to 300 ng target DNA/μL whole blood) may restrict signal production, the sensitivity of the anti-dsDNA EIA was evaluated with our standard method of detection using TBE-PAGE.

Materials and Methods

Materials

Acrylamide and N,N'-methylenediacrylamide were of electrophoretic grade.* Both PCR reagents and carryover prevention kit were used as supplied by the manufacturer.† Oligonucleotide primers were synthesized by automated phosphoramidite chemistry.‡ Unless specified, all other reagents were of the highest quality available.§

* Cat. No. 100-133 and 100-140, Boehringer Mannheim Corp., 9115 Hague Road, PO Box 50414, Indianapolis, IN 46250.
† Cat. No. N801-0055 and N808-0068, Perkin/Elmer Corp., 761 Main Avenue, Norwalk, CT 06859.
‡ Cyclone DNA Synthesizer, Milligen/Biosearch, Inc., New Brunswick Scientific Co., PO Box 4005, 44 Talmadge Road, Edison NJ 08818.
§ Sigma Chemical Co., PO Box 14508, St. Louis, MO 63179.
### TABLE I

**Oligonucleotide Sequences**

<table>
<thead>
<tr>
<th>Oligonucleotide Sequence</th>
<th>Strand</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Primer 1: 5’-GTTTTCCTGGATTATGCTGGGCAC-3’</td>
<td>(forward, coding)</td>
<td><em>Science</em> 1989;245:1073-80</td>
</tr>
<tr>
<td>Primer 2: 5’-GTTGGCATGCTTTGATGACGCTTC-3’</td>
<td>(reverse, noncoding)</td>
<td></td>
</tr>
<tr>
<td>Primer 3: 5’-GCAGAGTAGCTGAAACAGGA-3’</td>
<td>(forward, coding)</td>
<td><em>Proc Natl Acad Sci</em> 1990;87:8447-51</td>
</tr>
<tr>
<td>Primer 4: 5’-CATTCACAGTAGCTTACCCA-3’</td>
<td>(reverse, noncoding)</td>
<td></td>
</tr>
<tr>
<td>Probe N: 3’-CTTTTATAGTAGAAACCAC-5’*</td>
<td></td>
<td><em>Molec Cell Probes</em> 1991;5:459-66</td>
</tr>
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</table>

*Underlined triplet designates 3 bp deletion in M probe.*

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**PCR AMPLIFICATION**

Circular punches (3 mm diameter) from Guthrie card bloodspots were cut into 4 equal size wedge-shaped pieces with a razor blade. Each wedge was incubated with 1 mL distilled-deionized water twice for 30 min (with several inversions) to remove endogenous whole blood PCR inhibitors.* The washed one-quarter bloodspot was placed directly into a 0.5 mL PCR reaction tube and 33 μL of ultrapure water was added. The material was overlaid with 50 μL mineral oil and heated in a PCR thermal cycler† (95°C, 5 min). Following cooling to 25°C, 17 μL PCR reaction mix was added to achieve final concentrations of Tris-HCl, pH 8.3 (10 mmol/L), KCl (50 mmol/L), deoxyribonucleotide triphosphates (dATP, dUTP, dCTP, and dGTP, 200 μmol/L each), MgCl₂ (3 mmol/L), Taq polymerase (1.25 U), uracil N-glycosylase (0.5 U), gelatin (100 mg/L) and primers 1 and 2 or primers 3 and 4 (100 ng each primer)¹,²,⁷ (table I). For carryover prevention, the reaction mix was heated at 37°C and 94°C (10 min each) to activate and inactivate uracil N-glycosylase. PCR amplification consisted of denaturation (94°C, 1 min), annealing (55°C, 1 min), and elongation (72°C, 2 min) for 35 cycles. A final elongation was performed at 72°C for 20 min. Expected sizes of PCR products using primer set 1/2 and 3/4 are shown for the three genotypes (table II).

**EIA**

The anti-dsDNA enzyme immunoassay (EIA) was performed essentially as described by the manufacturer.** All reagents were allowed to come to room temperature prior to use. Briefly, PCR

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¹ NDC No 0574-0618-16, Paddock Laboratories, Inc., Minneapolis, MN 55427.

‡ Original Model, Perkin/Elmer Corp., 761 Main Avenue, Norwalk, CT 06859.

** GEN-ETI-K DEIA, Cat. No. PS0005, Sorin Biomedica, S.p.A., 13040 Sluggia (VC), Italy, distributed by INCASTAR Corp., 1990 Industrial Boulevard, PO Box 286, Stillwater, MN 55082.
TABLE II
Amplification Products

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Normal</th>
<th>Cystic Fibrosis Polymerase Chain Reaction Products (bp)</th>
<th>Heterozygote</th>
<th>Homozygote</th>
</tr>
</thead>
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<tr>
<td>1 and 2</td>
<td>98/98</td>
<td>98/95</td>
<td>95/95</td>
<td></td>
</tr>
<tr>
<td>3 and 4</td>
<td>491/491</td>
<td>491/488</td>
<td>488/488</td>
<td></td>
</tr>
</tbody>
</table>

products were denatured to single-stranded molecules in a boiling water bath (15 min) and immediately placed in an ice water bath for 15 min to prevent annealing. Hybridization buffer (100 µL) and denatured samples (20 µL) were added to microtiter plate wells coated with normal (N) or mutant (M) single-stranded cDNA probes (table I) for the ΔF508 mutation. Hybridization was performed for 1 h at 45°C in a thermostatically controlled water bath.†† The wells were washed three times with phosphate buffered saline containing Tween-20 (wash solution). Anti-dsDNA solution (100 µL) was added and the incubation continued (1 h, 25°C). The wells were washed and enzyme tracer solution (horseradish peroxidase-conjugated antimouse IgG, 100 µL) was added (1 h, 25°C). The wells were washed and chromogen/substrate solution (tetramethylbenzidine, H₂O₂, 100 µL) was added (30 min, 25°C). Following addition of stop solution (1 N H₂SO₄, 200 µL) the microtiter plates were scanned by a microtiter plate reader‡‡ at 450 nm. Absorbance results were corrected for background (650 nm).

TBE-PAGE

Tris-borate-EDTA/polyacrylamide gel electrophoresis (TBE-PAGE) was performed as described.8,9 Briefly, PCR samples (20 µL) were mixed with 6 µL sample buffer containing bromophenol blue (2.5 g/L) and Ficoll-type 400 (150 g/L). Samples (15 µL) were electrophoresed on 8 percent and 4 percent mini-polyacrylamide gels§§ (0.75 mm thickness) in the presence of 1X TBE at 75 V (constant voltage)¶¶ for 1 h (491 bp amplicons) and 2.5 h (98 bp amplicons), respectively (25°C). Gels were stained with ethidium bromide (0.5 µg/mL, 100 mL) for 10 min at 25°C on a rotatory platform (80 rev/min) and photographed with background ultraviolet illumination.¶¶ Electrophoretic migration of PCR products was compared relative to 123 bp DNA molecular weight ladders.* Normal and homozygote ΔF508 mutation were confirmed by mixing experiments in which normal control PCR product (10 µL) was added to sample (10 µL) prior to electrophoresis.

Results

TBE-PAGE ΔF508

GENOTYPE IDENTIFICATION

Following PCR amplification and prior to anti-dsDNA EIA, all Guthrie card

†† Model 25 (Precision Scientific), Varlen Instruments, Inc., NA, PO Box 2129, Joliet, IL 60434.
‡‡ Thermomax, Molecular Devices, Inc., 1311 Orleans Drive, Sunnyvale, CA 94089.
§§ Mini-PROTEAN II, Cat. No. 165-2940, Bio-Rad Laboratories, Life Science Group, 2000 Alfred Nobel Drive, Hercules, CA 94547.
¶¶ Model PS500X, Hoefer Scientific Instruments, 654 Minnesota Street, Box 77387, San Francisco, CA 94107.
* Cat. No. 15613-029, Gibco-BRL Laboratories, Inc., PO Box 68, Grand Island, NY 14072.
bloodspots were genotyped for the ΔF508 mutation by 8 percent TBE-PAGE. Amplicons obtained under standard PCR conditions from normal (lane 1), heterozygous (lane 2), and homozygous ΔF508 individuals (lane 3) were resolved by TBE-PAGE (figure 1A). Electrophoretic analysis of PCR products obtained from normal and homozygous ΔF508 samples results in the production of a single ethidium bromide-stained band (lanes 1 and 2). The lower position (i.e., higher electrophoretic mobility) of PCR product in lane 2 is suggestive of smaller size. However, unequivocal identification is confirmed by mixing with PCR product from a known normal control (98 bp) prior to electrophoresis (lanes 1+ and 2+). Mixing results in the generation of two ethidium bromide-stained bands (98 and 95 bp) for homozygous ΔF508 mutation (lane 2+) whereas a single band (98 bp) is observed for the normal genotype (lane 1+).

In comparison, the presence of two PCR products (98 and 95 bp amplicons) and heteroduplex formation (lane 3) designates this individual as heterozygous. As expected, the intensity of the ethidium bromide-stained bands obtained from the heterozygote (lane 3) is approximately one-half that from normal (lane 1) or homozygous individuals (lane 2). This lower intensity of heterozygote PCR products results in obscuring of the 95 bp band after mixing (lane 3+). In contrast, when identical bloodspots were amplified under modified PCR conditions (491 bp amplicons), TBE-PAGE was unable to genotype unequivocally the PCR products obtained from the normal (lanes 1 and 1+) or homozygous ΔF508 (lanes 2 and 2+) sample (figure 1B). Heteroduplex formation, however, is clearly evident in the heterozygote with (lane 3+) and without mixing (lane 3).

**EIA ΔF508 Genotype Identification**

Binding of amplification products obtained under standard PCR conditions (98 bp amplicons) from normal, heterozygous, and homozygous ΔF508 individuals was highly specific to corresponding cDNA probes (table III). The PCR product from normal individuals resulted in approximately 9-fold higher binding to normal (N) cDNA probe versus the mutant (M) cDNA probe. Approximately 30-fold higher binding was observed for M probe versus the N probe when PCR products were analyzed from individuals

![Figure 1. TBE-PAGE of PCR amplification products for the CF ΔF508 mutation. Electrophoresis was performed on (A) 8 percent polyacrylamide for 98 bp amplicons (primer set 3/4) and (B) 4 percent polyacrylamide for 491 bp amplicons (primer set 1/2). Lane: 1, normal; 2, homozygous ΔF508; 3, heterozygous ΔF508. Plus sign (+) indicates sample mixed with amplicons from normal control prior to electrophoresis. Hd, heteroduplex; Mw, 123 bp DNA ladder.](image-url)
Results of Enzyme Immunoassay

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Mean Absorbance</th>
<th>Mean N/M</th>
<th>SD</th>
<th>(n)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Probe</td>
<td>M Probe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.051</td>
<td>0.130</td>
<td>8.793</td>
<td>5.419</td>
<td>5</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0.595</td>
<td>0.909</td>
<td>0.636</td>
<td>0.152</td>
<td>19 pc &lt;0.0001</td>
</tr>
<tr>
<td>Homozygous</td>
<td>0.044</td>
<td>1.399</td>
<td>0.036</td>
<td>0.017</td>
<td>3 p&lt;0.05</td>
</tr>
</tbody>
</table>

*Versus normal N/M (unpaired two-tailed t test).

Slightly higher activity was detected with the M (0.909) versus N (0.595) probes when PCR products were analyzed from heterozygous samples. This difference, however, was not significant (p > 0.05, paired t test). Reduction of data to N/M ratios resulted in mean values of 8.8, 0.6, and 0.04 for normal, heterozygous, and homozygous samples (Table 3). The large standard deviation (SD) for normal N/M ratio results from the fact that these values extended over a wide range (5.036 to 17.158). A similar high SD (relative to mean N/M ratio) was also observed with homozygous CF samples (N/M ratio range 0.026–0.055).

Statistical evaluation of this data indicated that the hybridization signal for heterozygote and homozygote ΔF508 mutation were significantly different from normal (table III). Despite the considerable variation in observed N/M ratios (2- to 3-fold), comparison to the recommended N/M cut-off values resulted in 100 percent correlation to TBE-PAGE results for all three genotypes (table IV).

EIA vs TBE-PAGE

The sensitivity of the anti-dsDNA EIA was compared to TBE-PAGE. As can be seen, the ability to visually detect ethidium bromide-stained PCR products substantially decreased at serial dilutions greater than 1:5 for normal and homozygote ΔF508 PCR products (figures 2A and 2C). Owing to their lower concentration

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed Range (N/M)</th>
<th>Recommended* Cut-off Range</th>
<th>vs TBE-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5 – 17</td>
<td>&gt;2</td>
<td>100%</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0.5 – 1.1</td>
<td>0.3 – 2</td>
<td>100%</td>
</tr>
<tr>
<td>Homozygous</td>
<td>0.03 – 0.05</td>
<td>0 – 0.3</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Recommended by the manufacturer.
DETECTION OF CYSTIC FIBROSIS ΔF508 MUTATION

Figure 2. Sensitivity of ethidium bromide-stained TBE-PAGE. Electrophoresis was performed on 8 percent polyacrylamide for serial dilutions of 98 bp amplicons (primer set 3/4) for normal (A), heterozygote (B) and homozygote CF ΔF508 mutation (C). Lane 1 and 8, heterozygote control; 2, not diluted; 3, 1:2; 4, 1:5; 5, 1:10; 6, 1:20; 7, 1:50. Hd, heteroduplex.

(approximately one-half), heterozygote PCR products were virtually nonexistent at dilutions greater than 1:2 (figure 2B). Dilutions of PCR products greater than 5-fold resulted in no detectable ethidium bromide-stained bands for any of the three amplified genotypes. In contrast, good quantitation of EIA absorbance values for N and M cDNA probes was obtained over a wide dilution range for normal, heterozygote and homozygote ΔF508 PCR products (figure 3A–C). Data reduction to N/M ratios over this concentration range resulted in the accurate detection of the three genotypes except for the highest-diluted normal PCR product (figure 3D–F). The 50-fold diluted normal N/M ratio was slightly (5 percent) below the cutoff (1.914 vs 2).

Analysis time for TBE-PAGE was found to be substantially shorter (25 percent) than the EIA (3.5–4 h and 4.5–5 h,
FIGURE 3. Sensitivity of anti-dsDNA EIA. PCR products from normal (A), heterozygote (B) and homozygote ΔF508 mutation (C) were assayed following serial dilution (1 to 50-fold). Absorbance values shown on left. Data reduction to N/M ratios and established reference intervals shown on right.
DETECTION OF CYSTIC FIBROSIS ΔF508 MUTATION

respectively) when small numbers of samples (n = 10) were analyzed. However, TBE-PAGE was not a convenient method for screening large numbers of PCR samples (n > 15).

Discussion

Excellent correlation (100 percent) was found between EIA and TBE-PAGE for PCR products obtained from normal, heterozygous, and homozygous ΔF508 individuals using standard (98 bp amplicons) and modified (491 bp amplicons) amplification conditions. Under the hybridization conditions employed (45°C) little nonspecific binding to cDNA probes was observed. The EIA reactivity was not influenced by amplicon size because comparable results were obtained with either the 98 or 491 bp amplicons. Similar observations for the anti-dsDNA EIA have been previously reported for PCR products encompassing the CF G542X (425 bp) and ΔF508/ΔI507 (97 bp) mutations. In contrast to EIA, TBE-PAGE was unable to unequivocally resolve larger amplification products for the ΔF508 mutation despite mixing experiments. Because of inherently low resolving power, TBE-PAGE alone is not useful for accurate identification of genetic defects especially those which result from single bp (i.e., point) mutations.

Previous work with the anti-dsDNA EIA for the CF ΔF508 mutation involved the detection of PCR amplified sequences from highly purified genomic material. These investigators suggested that target copy number was a critical factor for EIA sensitivity. Although the entrapped Guthrie card DNA in our amplification protocol represents less than 1 μL of whole blood (National Committee for Clinical Laboratory Standards, 1985), it was demonstrated by us that this amount of genomic material was sufficient for generating quantifiable levels of amplicons for EIA over a 50-fold concentration range. Therefore, elaborate and time-consuming DNA purification-extraction protocols which can involve proteinase K digestion, detergent treatment, and phenol-chloroform extraction are not required for amplification of Guthrie card samples. The high sensitivity of the anti-dsDNA EIA should make it feasible to generate sufficient amplicon concentration in smaller reaction volumes, thereby decreasing PCR reagent costs.

Guthrie card specimens collected from neonates represent an important yet relatively underutilized source of DNA for genetic screening. The utility of an anti-dsDNA EIA microtiter plate method is demonstrated for detection of the CF ΔF508 mutation following amplification of Guthrie card DNA. The EIA method was more sensitive than our routine method of detection (TBE-PAGE) and generated quantitative results over a wide concentration range of PCR products. In addition, EIA was not dependent on the subjective interpretation of ethidium bromide-stained bands or autoradiographs obtained from Southern or dot blots. The anti-dsDNA EIA does not require extensive reagent preparation and avoids the use of toxic reagents such as acrylamide and ethidium bromide. Although method performance time was slightly longer for small numbers of samples, the microtiter EIA should be readily adaptable for large-scale automated routine screening purposes. Thus, EIA based on anti-dsDNA makes this nonradioactive technology widely applicable for any ASO hybridization method.

References


