We describe a simple hybridization assay performed in microtitration wells with use of DNA probes labeled with three different lanthanide chelates for detection of seven mutations that cause cystic fibrosis. The assay is based on DNA amplification of four fragments containing the mutations (ΔF508, G1717→A, G542X, R553X, 3905 insertion T, W1282X, and N1303K) by PCR, followed by hybridization with short, allele-specific oligonucleotide probes labeled with europium, terbium, or samarium chelates. Because the technology makes it possible to hybridize three DNA probes simultaneously in one reaction, all 14 mutation-related alleles were detected in a total of five reaction wells. Blood spot specimens, obtained from children with cystic fibrosis, their parents, and their siblings, have been assayed, and for all the probes the positive signal-to-noise ratios are >10. Solution hybridization utilizing triple-label time-resolved fluorometry combined with PCR is a suitable procedure for large-scale screening and automation.

INDEXING TERMS: allele-specific hybridization • PCR • hybridization assay • genetic disease • lanthanide chelate

Cystic fibrosis (CF) is one of the most common autosomal genetic disorders in Caucasian populations, with a carrier frequency of 1 in 25, affecting about 1 in 2500 individuals. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and the isolation and cloning of the CFTR gene [1–3] have created the basis for the development of DNA tests used for genotyping fetuses and screening for carriers. More than 230 CFTR mutations have been associated with disease, many of them relatively rare [4], making the genetic screening complicated. Quantitation of immunoreactive trypsin (IRT) has been used in primary screening for CF, followed by genetic examination of samples with increased IRT concentrations [5]. The mutation ΔF508 is widely used for screening because it is the most frequent CFTR mutation. However, in some countries the frequency is much less than 80% [6], making the ΔF508 screening inadequate.

The diagnostics of human genetic diseases has been revolutionized by PCR [7]. Several technologies have been used in detection of the amplified target sequences, such as allele-specific hybridization of PCR product with microtitration plates [8] or membranes [9] as solid support, oligonucleotide ligation assay [10], or allele-specific PCR [11]. Automated techniques are needed to exploit the possibilities offered by molecular methods. Improved DNA techniques are required in screening programs to attain high test throughputs simultaneously with appropriate assay properties, such as technical reliability, detection limits, specificity, and feasibility for automation.

Time-resolved fluorometry utilizes the long-lived fluorescence of rare earth metals, lanthanides [12]. The fluorescence of lanthanides, e.g., europium (Eu), terbium (Tb), and samarium (Sm), can be measured after a time delay when the short-lived background fluorescence of biological material has decayed. In addition, the emission peaks of different lanthanides are clearly distinguishable because they are sharp and occur at different wavelengths, making the simultaneous measurement possible. Chelates of lanthanides can be used as labels in various applications without changing the properties of bioaffinity molecules (for reviews, see Soini and Lövgren [13] and Gudgin Dickson et al. [14]).

The aim of our study was to develop a user-friendly hybridization assay for seven frequent CFTR mutations. We utilized triple-label time-resolved fluorometry and short, allele-specific oligonucleotide probes to detect ΔF508, G1717→A, G542X, R553X, 3905 insertion T
(3905insT), W1282X, and N1303K mutations after PCR amplification of the CFTR gene exons 10, 11, 20, and 21.

**Materials and Methods**

**REAGENTS AND INSTRUMENTATION**

DNA synthesis reagents, including calibrated phosphoramidites and biotin amidite and an ABI 392 DNA/RNA synthesizer, were supplied by Applied Biosystems, Inc. (Foster City, CA). DNA polymerase and reaction buffer for amplification were purchased from Finnzymes Oy (Espoo, Finland), dNTPs and Phast electrophoresis system and reagents from Pharmacia Biotech (Uppsala, Sweden), and Perkin-Elmer DNA Thermal Cycler from Perkin-Elmer (Norwalk, CT). Diaminohexane-modified deoxycytidine phosphoramidite (modC), active lanthanide chelates, streptavidin-coated microtitration plates, DELFIA Assay Buffer, Wash Solution, and Enhancement Solution, and a DELFIA Plateshake, Platewash, Plate Dispense, and 1234 Research Fluorometer were from Wallac Oy (Turku, Finland). All other reagents used were of analytical grade.

**SAMPLES**

Twelve samples of purified DNA obtained for sequencing purposes from known CF patients were analyzed to validate the detection probes. Eighty blood spot samples were used to verify the extraction of DNA from blood spots and to validate the whole methodology. Blood samples, obtained from children with CF, their parents, and their siblings, were spotted on filter paper cards and dried at room temperature (RT). The samples were kept refrigerated at 4 °C and moisture-free until analyzed.

**SAMPLE PREPARATION**

Twelve purified and sequenced DNA samples were diluted into 10 mmol/L Tris-HCl, pH 7.5, 50 μmol/L EDTA to a final concentration of 200 ng/μL. A 1.5-μL sample was taken into the PCR reaction. For analysis of the mutations in a dried filter paper blood specimen, a 3-mm disk was punched and treated according to liitia et al. [15]. The disk was boiled in alkaline solution to liberate DNA, and the solution was neutralized and centrifuged for 5 min at 16 000g. Pretreated samples were stored at −20 °C. A 3-μL sample was taken from the supernatant into the PCR reaction.

**OLIGONUCLEOTIDE SYNTHESIS**

The oligonucleotides (for primers and probes, see Table 1) were synthesized by an ABI 392 DNA/RNA synthesizer and phosphoramidite chemistry. ModC [16] was used to introduce amino functions into the detection probes for labeling. Twenty modCs were assembled to the 5’ end of probes by an accepted coupling procedure. Biotin amidite was used to introduce the biotin (Bio) to the 5’ end of certain PCR primers and to the 3’ end of the synthetic targets according to the manufacturer’s instructions. The synthetic targets were assembled in such a way that a certain target oligonucleotide consisted of sequences complementary to those detection probes hybridized in a certain well. The sequences of the synthetic targets from the 5’ end to the 3’ end were as follows: AGTAATAGCATCCTTCTCAAGAACGT (Bio (well 1 target), CACCAAGATGATCCCTCTCAAGAACGT (well 2 target), AACCAATGATATGAGATGTCATCAGGCGAAG Bio (well 3 target), TCTCAAGAACTTTTTTBio (well 4 target), and AGCTTTTTTGTACACTTTCTCCTAGT (well 5 target). The cross-reaction targets contained mutated sequences of the wild-type targets and vice versa. Oligonucleotides were purified by polyacrylamide urea gel electrophoresis by accepted methods.

**Table 1. Sequences, labels, and PCR reaction composition for primers.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Wild/ mutant</th>
<th>PCR reaction no.</th>
<th>5'-Primer</th>
<th>3'-Primer</th>
<th>No. of hybridization wells</th>
<th>Detection probe 5'-3' sequence and label</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>w</td>
<td>1</td>
<td>AAGCACAGTGGAGAATTTTC</td>
<td>BioCTCTTCTAGTGGCATGCT</td>
<td>2</td>
<td>Tb-(modC)_{20}ATCATTTTGGT</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G^{177→A}</td>
<td>w</td>
<td>1</td>
<td>BioGAGCATCTAAAGTGACTC</td>
<td>BioCATGAATGACATTTACAGCAA</td>
<td>3</td>
<td>Sm-(modC)_{20}TATCATTTGGT</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G542X</td>
<td>w</td>
<td>1</td>
<td>BioGAGCATCTAAAGTGACTC</td>
<td>BioCATGAATGACATTTACAGCAA</td>
<td>2</td>
<td>Eu-(modC)_{20}ATGCTTTTAGTAC</td>
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<td></td>
<td>m</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R553X</td>
<td>w</td>
<td>1</td>
<td>BioGAGCATCTAAAGTGACTC</td>
<td>BioCATGAATGACATTTACAGCAA</td>
<td>1</td>
<td>Tc-(modC)_{20}GTTGAGGAAAGGATCCAAG</td>
</tr>
<tr>
<td>N1303K</td>
<td>w</td>
<td>2</td>
<td>AAGTATTTATTTCTGGAACAT</td>
<td>BioTTCCTGATCCTACACTGGTT</td>
<td>2</td>
<td>Sm-(modC)_{20}ACATTGTAAGGAAAGGATCCAAG</td>
</tr>
</tbody>
</table>

Sequences, labels, and design of hybridization wells for wild-type specific (w) and mutant specific (m) detection probes for seven CFTR mutations. Underlining indicates the point of mutation, ∧ the location of the 3-bp deletion in mutation ΔF508, and * the point where T is inserted in mutation 3905insT.
LABELING OF OLIGONUCLEOTIDES
The detection probes were labeled with the Eu(III), Tb(III), and Sm(III) chelates of 2,2’,2”-tris[bis(ethyl-
phosphono)methyl]tetakis(acetic acid) or 2,2’-tris[bis-
(4-phenylethynylethyl)pyridine-2,6-diyli)bis(methylene-
nitrito)tetrakis(acetic acid) [17]. The labeling and purification were performed as described earlier [8] with some modifications. The active chelate of Eu, Tb, or Sm was dissolved in 50 mmol/L NaHCO₃/
Na₂CO₃ buffer, pH 9.6, to a concentration of 30
mmol/L. An aliquot of 50 μg of oligonucleotide was
dried down and a 12-fold molar excess of chelate (compared with the amino groups) was added. The reaction mixture was incubated overnight at RT. The oligonucleotide was prepurified by ethanol precipitation by accepted methods. The precipitate was dis-
dissolved in 100 μL of 10 mmol/L Tris-HCl, pH 7.5, 50
mmol/L NaCl, 50 μmol/L EDTA (elution buffer) and loaded on a Sephadex G-50 DNA grade column (Phar-
macia Biotech). The labeling degree of the detection probes was measured according to Dahlén et al. [8]. The probes contained 6–19 lanthanides/oligonucleotide.

PCR
The principle of the assay is presented in Fig. 1. Two reactions were performed to amplify the fragments in exons 10 and 11 (PCR1) and in exons 20 and 21 (PCR2). Both reactions contained two sets of primers (Table 1). The PCR amplification was performed in 1× reaction buffer (10 mmol/L Tris-HCl, pH 8.8, 50 mmol/L KCl, 2.5
mmol/L MgCl₂, 1 mL/L Triton X-100) containing 200
μmol/L each dNTP, 0.1 μmol/L each primer, and 1 U of DynaZyme™ II DNA Polymerase. A 3-
μL sample of pretreated blood spot specimen or 300 ng of isolated DNA
was added to 100 μL of reaction mixture. In each exper-
iment 300 ng of chromosomal DNA was added as a positive control. Also to control the contamination in PCR a 3-μL aliquot of water was added instead of sample. Amplification was carried out for 30 cycles in a Perkin-
Elmer DNA Thermal Cycler with the following cycle program: 1 min at 95 °C, 1 min at 56 °C, and 1 min at 74 °C.

SOLUTION HYBRIDIZATION
Analysis of the amplified DNA fragments was performed by collecting the biotinylated fragments, denaturing the collected fragments, and hybridizing them with lan-
thanide-labeled probes (Fig. 1). First, 15 μL of the ampli-
fied PCR product in duplicate were collected onto strepta-
vidin-coated microtiritation wells in 50 μL of reaction buffer (DELFIA Assay Buffer supplemented with NaCl and Tween 20 to final concentrations of 1 mol/L and 1
mL/L, respectively). In each experiment 3 × 10¹⁰ molec-
ules of the synthetic target or cross-reaction target per well was added as the hybridization and cross-reaction control. Wells containing only buffer instead of sample were included to control the hybridization background. Collection was carried out at RT with shaking on a DELFIA Platemake for 30 min. Uncollected fragments were removed by three washings at RT with DELFIA Wash Solution in a DELFIA Platewash.

Before hybridization, the double-stranded products were melted in 150 μL of 50 mmol/L NaOH per well at RT with shaking for 5 min. After denaturation, uncol-
lected single-stranded DNA molecules were washed away as described above.

The collected DNA molecules were hybridized with the allele-specific lanthanide-labeled detection probes. The fragments amplified in PCR1 were analyzed with

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**Table 2. Hybridization characteristics of detection probes determined with synthetic target oligonucleotides.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Wild/mutant</th>
<th>No. and type of label</th>
<th>Optimal hybridization temperature, °C</th>
<th>Optimal probe conc., ng/well</th>
<th>Hybridization sensitivity (target molecules/well)</th>
<th>Cross-reactivity, %</th>
<th>Maximal hybridization efficiency, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508</td>
<td>w</td>
<td>19Tb</td>
<td>31</td>
<td>2</td>
<td>4.1 × 10⁷</td>
<td>0.0</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>19Sm</td>
<td>24</td>
<td>5</td>
<td>1.2 × 10⁸</td>
<td>1.0</td>
<td>13.5</td>
</tr>
<tr>
<td>G¹⁷⁷⁶→A</td>
<td>w</td>
<td>18Eu</td>
<td>17</td>
<td>5</td>
<td>5.9 × 10⁷</td>
<td>0.2</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>19Tb</td>
<td>&lt;10</td>
<td>5</td>
<td>5.6 × 10⁷</td>
<td>0.1</td>
<td>8.0</td>
</tr>
<tr>
<td>G542X</td>
<td>w</td>
<td>18Eu</td>
<td>18–27</td>
<td>5</td>
<td>6.7 × 10⁷</td>
<td>0.4</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>19Tb</td>
<td>20</td>
<td>10</td>
<td>8.1 × 10⁷</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>R553X</td>
<td>w</td>
<td>9Sm</td>
<td>32</td>
<td>2</td>
<td>3.2 × 10⁸</td>
<td>1.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>16Eu</td>
<td>22</td>
<td>1</td>
<td>2.2 × 10⁷</td>
<td>1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>3905insT</td>
<td>w</td>
<td>18Tb</td>
<td>25</td>
<td>5</td>
<td>1.8 × 10⁷</td>
<td>2.9</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>19Sm</td>
<td>22</td>
<td>2</td>
<td>1.8 × 10⁷</td>
<td>1.0</td>
<td>21.0</td>
</tr>
<tr>
<td>W1282X</td>
<td>w</td>
<td>19Eu</td>
<td>22</td>
<td>1</td>
<td>3.2 × 10⁷</td>
<td>0.4</td>
<td>14.0</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>19E</td>
<td>20</td>
<td>2</td>
<td>1.7 × 10⁷</td>
<td>0.5</td>
<td>21.0</td>
</tr>
<tr>
<td>N1303K</td>
<td>w</td>
<td>9Sm</td>
<td>31</td>
<td>10</td>
<td>6.4 × 10⁷</td>
<td>0.6</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>13Tb</td>
<td>27</td>
<td>2</td>
<td>3.1 × 10⁷</td>
<td>0.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*a* For sequences, see Table 1.

*b* Determined using 1 × 10¹⁵ target molecules/well.

*c* Defined from calibration curve as target concentration corresponding to 3 × SD of background signal.

*d* Determined using optimal probe concentrations.
probes specific for ΔF508, G1717→A, G542X, and R553X in wells 1–3, and the fragments amplified in PCR2 with probes specific for 3905insT, W1282X, and N1303K in wells 4 and 5 (Fig. 1). The probe mixtures contained optimal concentrations of each labeled probe (Table 2). Hybridization was carried out in 100 μL of reaction buffer (described above) at RT with shaking for 2 h. After hybridization, the wells were washed six times at RT with DELFIA Wash Solution.

For detection, 200 μL of DELFIA Enhancement Solution per well were added by a DELFIA Plate Dispense, and the wells were incubated at RT on a Plateshake for 15 min. The Eu and Sm fluorences were measured in a 1234 DELFIA Research Fluorometer. After Eu and Sm measurement, Tb was enhanced by adding 50 μL of Tb Enhancement Solution (research reagent, Wallac Oy). The wells were shaken briefly and allowed to stand for 2 min before the Tb fluorescence was measured in the 1234 DELFIA Research Fluorometer.

**Results**

**ASSAY DESIGN AND DNA AMPLIFICATION**

The assay was constructed to analyze seven mutations that cause CF. The mutations of interest were ΔF508 in exon 10, G1717→A, G542X, and R553X in exon 11, 3905insT and W1282X in exon 20, and N1303K in exon 21. The mutation regions in each exon were amplified in one fragment, resulting in four amplification products. Because both wild-type probes and mutant probes are used in allele-specific hybridizations, altogether 14 detection probes were needed. As the technology made it possible to measure three labels in one well, the 14 detection probes could be divided into five wells.

During optimization of PCR conditions, the quantity of the four PCR products was estimated by Phast gel electrophoresis. When all the fragments were amplified in one multiplex PCR with four primer pairs, the product concentrations were low compared with single PCR. Because single PCR reactions, i.e., four separate amplifications, would have been impractical, we decided to divide the amplifications into two different reactions. Amplification of two products in one reaction, within exons 10 and 11 (PCR1) and exons 20 and 21 (PCR2), had no remarkable influence on the concentration of the final product.

The annealing temperature, concentration of primers, and amount of DNA polymerase in the amplification reaction were optimized. For annealing temperature, 56 °C was found to be the most convenient compromise. A primer concentration of 0.1 μmol/L and 1 U of DNA polymerase per reaction were found to be adequate in obtaining the highest amount of products.

The amplification product from PCR1 (exons 10 and 11) was divided into three duplicate wells because the four mutations of interest are located within these exons; i.e., eight detection probes were hybridized with the product (for assay construction, see Fig. 1 and Table 1). The PCR product from PCR2 (exons 20 and 21) was divided into two duplicate wells and hybridized with six detection probes. The hybridization reactions were distributed as follows: wild-type G1717→A probe and mutant G542X probe were hybridized in well 1, wild-type probes for ΔF508, G542X, and R553X in well 2, and mutant probes for ΔF508, G1717→A, and R553X in well 3. The detection probes for the mutations in exon 20 and 21 were hybridized in well 4 (wild-type probes) and in well 5 (mutant probes). Typically, for example, wild-type G1717→A samples gave Eu signal in hybridization well 1, and mutant...
G$^{1717\rightarrow A}$ samples gave Tb signal in well 3. Carrier samples gave Eu and Tb signals in wells 1 and 3, respectively.

**Optimization of Hybridization**

Biotinylated synthetic targets containing sequences complementary to the detection probes were used to optimize the hybridization conditions. The collection kinetics of biotinylated target DNA onto streptavidin-coated wells was studied. The reactions were performed at RT with shaking, and hybridizations were performed for 2.5 h. The collection reaction was completed in 15 min, but we decided to use 30-min collections in subsequent reactions. For optimization of the hybridization time, the detection probes were hybridized at RT with shaking for various incubation times. No substantial increase in the hybridization signals was obtained after a 2-h hybridization (data not shown).

The optimal probe concentration was studied by monitoring the ratio of specific and unspecific signals, i.e., signal-to-noise ratio at various probe concentrations with the fixed amount of synthetic target DNA ($1 \times 10^{11}$ molecules per well). The hybridizations were performed at RT with shaking for 2 h. The optimal concentration of probes varied from 1 to 10 ng of probe per well (Table 2). The hybridization efficiency with different concentrations of probes was studied by measuring the signal of each probe before and after hybridization with $1 \times 10^{11}$ target molecules per well. The highest hybridization efficiency was typically obtained with probe concentrations lower than 1 ng/well. Fig. 2 shows typical signal-to-noise ratios and hybridization efficiencies for the wild-type G$^{1717\rightarrow A}$ probe at different probe concentrations. The maximal efficiency varied from probe to probe between 2.5% (the mutant G542X probe) and 38% (the mutant 3905insT probe) (Table 2).

To choose the hybridization temperature, we made a temperature profile of each probe. The lowest optimal hybridization temperature was $<10^\circ$C (the mutant G$^{1717\rightarrow A}$ probe), and the highest optimum was 32 $^\circ$C (the mutant R553X probe) (Table 2). The hybridization temperature was chosen by comparing the relative hybridization signals of each probe at different temperatures. For all the probes the highest average and the lowest variation between the relative signals were achieved between 18 and 23 $^\circ$C (Table 3). The same fact can be seen in Fig. 3, showing the temperature vs relative hybridization signal profiles of three probes hybridized in one well. For practical reasons, subsequent hybridizations were performed at RT.

### Table 3. Average of relative hybridization signals of all 14 detection probes calculated from hybridizations carried out at different temperatures.

<table>
<thead>
<tr>
<th>Hybridization temperature, $^\circ$C</th>
<th>Average of hybridization signals, % of maximal signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>80 $\pm$ 17</td>
</tr>
<tr>
<td>14</td>
<td>82 $\pm$ 14</td>
</tr>
<tr>
<td>18</td>
<td>90 $\pm$ 9</td>
</tr>
<tr>
<td>23</td>
<td>92 $\pm$ 8</td>
</tr>
<tr>
<td>27</td>
<td>85 $\pm$ 14</td>
</tr>
<tr>
<td>33</td>
<td>68 $\pm$ 23</td>
</tr>
<tr>
<td>38</td>
<td>33 $\pm$ 19</td>
</tr>
</tbody>
</table>

![Graph](image1.png)  
**Fig. 2.** Signal-to-noise ratio and hybridization efficiency of wild-type G$^{1717\rightarrow A}$ probe.  
The signal-to-noise ratio ($\frac{\text{hybridization signal}}{\text{nonspecific signal}}$) ($\bullet$) and the hybridization efficiency (%) ($\frac{\text{signal after hybridization}}{\text{signal before hybridization}}$) ($\square$) at different probe concentrations.

![Graph](image2.png)  
**Fig. 3.** Hybridization temperature profiles of three detection probes hybridized in one well.  
Relative hybridization signals of mutant R553X ($\bullet$), G$^{1717\rightarrow A}$ ($\square$), and ΔF508 ($\diamond$) probes hybridized at optimal probe concentrations at different temperatures with $1 \times 10^{11}$ synthetic target molecules/well.
DETECTION LIMITS AND SPECIFICITY OF PROBES
Calibration curves with various concentrations of synthetic targets were determined to define the lowest detectable amount of synthetic target per well with optimal probe concentrations. A typical calibration curve for the wild-type G542X probe is shown in Fig. 4. The detection limit was defined as the background + 3SD of background. Thus the detection limits of the probes varied from $2 \times 10^7$ target molecules to $3 \times 10^8$ molecules (Table 2). The detection limits of Eu- and Tb-labeled probes were typically $\sim 5 \times 10^7$ target molecules, whereas that of Sm-labeled probes was $\sim 2 \times 10^8$ target molecules.

The cross-reactivity of wild-type probes was determined by hybridization with synthetic targets containing complementary sequences of mutant alleles, and mutant probes with complementary sequences of wild-type alleles with optimal probe concentrations. The cross-reactivity was typically very low, and only with high concentrations of target could the signal be distinguished from the background. With the highest target concentration used, the cross-reactivity was typically <1% of specific hybridization signal, except for the 3905insT mutation (Table 2), for which the cross-reactivity of the wild-type probe was $\sim 3\%$, and that of the mutant probe $\sim 6.5\%$. Fig. 4 illustrates the cross-reactivity of the wild-type G542X probe with different target concentrations.

VERIFICATION OF THE ASSAY
Purified DNA samples with known mutations were analyzed to verify the assay. The typical results from samples detected with all 14 probes and the interpretation of results are shown in Table 4. The signal-to-noise ratios were typically $>40$, but some probes had a signal-to-noise ratio as low as 10 with carrier samples. A signal-to-noise ratio of at least 10 was considered positive (Fig. 5). On the basis of this criterion, all the samples could be analyzed correctly. For verification of the assay with blood spots,

![Fig. 4. Calibration curve and cross-reaction of wild-type G542X probe.](Image)

**Amount of target DNA (molecules / $10^5$/well)**

Fig. 4. Calibration curve and cross-reaction of wild-type G542X probe. Hybridization signals of wild-type G542X probe hybridized with different concentrations of wild-type specific synthetic target ($\bullet$) and cross-reaction signals with mutant specific synthetic target ($\Delta$) (background subtracted). The detection limit ($7 \times 10^5$ target molecules/well) is defined from the calibration curve as the target concentration corresponding to $3 \times$ SD of background signal.

---

**Table 4. Results of DNA samples analyzed for seven CFTR mutations with 14 detection probes.**

<table>
<thead>
<tr>
<th>Signal-to-noise ratio for wild/mutant</th>
<th>$\Delta$F508</th>
<th>G$^{2717}$→A</th>
<th>G542X</th>
<th>R553X</th>
<th>3905insT</th>
<th>W1282X</th>
<th>N1303K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w</td>
<td>m</td>
<td>w</td>
<td>m</td>
<td>w</td>
<td>m</td>
<td>w</td>
</tr>
<tr>
<td>Purified DNA samples</td>
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</tr>
<tr>
<td>293</td>
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<td>255</td>
<td>1.1</td>
<td>65</td>
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<td>79</td>
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<td>31</td>
<td>61</td>
<td>2.4</td>
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Boldface numbers indicate signal-to-noise ratios considered positive.
amplified samples, each plotted for seven mutations, D
simultaneous monitoring of multiple genes, but it is not
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been applied to the DNA diagnostics, making the auto-
sible for automation. Capillary gel electrophoresis has
rather impractical methods when large batches of samples
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integration of this technique with present meth-
ods are laborious and reagent-consuming. Numerous CF
Discussion
CF is one of the most common severe inherited disorders
in Caucasian populations. CF is transmitted as an autosomal recessive disorder, and it is estimated that 5% of the population are carriers for one of the mutations causing CF [18]. In the current literature more than 450 mutations causing CF have been described. Some mutations are very frequent, e.g., ΔF508, whereas others have been described in one family only.
Although widely used, conventional screening meth-
ods are laborious and reagent-consuming. Numerous CF
screening studies for simultaneous detection of several mutations have been implemented over the past few years with reverse dot-blot analysis [19], a series of restriction enzyme cuts combined with dot blots [20], and denatur-
ing gradient gel electrophoresis [21, 22], all of which are rather impractical methods when large batches of samples are run. Because automation makes molecular diagnostics more productive, the screening technique should be feasi-
able for automation. Capillary gel electrophoresis has been applied to the DNA diagnostics, making the auto-
mation of the PCR product detection possible [23]. How-
ever, the integration of this technique with present meth-
ods used in clinical laboratories could be laborious. The
oligonucleotide array technology, which has also been used in hybridization experiments [24], has potential in simultaneous monitoring of multiple genes, but it is not
yet applicable in the clinical laboratory. In the time-
resolved fluorometric technique presented in this paper, the use of short lanthanide-labeled oligonucleotide probes functioning at RT is combined with the microtitration format, enabling automation of the procedure. The auto-
mation is facilitated by the fact that the data are produced in numerical form, thus making automatic handling of results possible. Time-resolved fluorometry is also feasible in other applications, making the IRT immunoassay compatible with genetic testing, for example.
Lanthanide chelates have been used as labels in the
simultaneous detection of two CFTR alleles from one hybridization reaction [25] and in triple-label hybridiza-
tions detecting two type 1 diabetes susceptibility, and two protective, alleles of the HLA-DQB1 gene [26]. We have developed a user-friendly triple-label hybridization assay for seven CFTR mutations with Eu, Tb, and Sm chelates as labels. The method is based on time-resolved fluorometry and PCR amplification. The amplified DNA is detected by simultaneous hybridization of three alleles per well, in a total of 14 hybridizations in five wells.
The mutations of interest exist in exons 10, 11, 20, and
21 of the CFTR gene. Four fragments were amplified to
detect seven mutations. As in numerous applications, multiplex PCR was performed to amplify the fragments. Because simultaneous amplification of all the fragments lowered the product concentrations, amplifications were performed in two separate reactions. Performance of two separate reactions was supported by the decrease in primer consumption, as two reactions would have been required anyway to produce material sufficient for five hybridization reactions in duplicate. In addition, two separate PCR reactions reduced the unnecessary use of streptavidin surface capacity by biotinylated PCR prod-
ucts and unreacted primers, because in total six biotiny-
lated primers were used in the amplification and at the most only three PCR products can be detected simulta-
aneously in one well (Fig. 1 and Table 1).
Earlier, a capture and reporter oligonucleotide system had been utilized for detection of ΔF508 by time-resolved fluorometry [25, 27]. In these studies, 14- and 21-nucleo-
tide-long allele-specific DNA probes, respectively, were
used, and the hybridizations and washes were performed at relatively high temperatures to obtain specificity. We have developed a hybridization assay that can be per-
formed at RT, thus making the assay easier to automate. The probes were designed to be short enough to attain specificity toward point mutations at RT. To improve the specificity, probes were chosen to originate from either
strand of the CFTR gene in such a manner that no G-T
mismatch resulted in cross-reaction. The cross-reactivity
of each probe was tested with synthetic oligonucleotide
targets and, when homozygote mutant DNA samples were available, also with PCR products. For all the probes, the signal of cross-reaction was measurable only when
3 × 10^{10}–1 × 10^{11} molecules of synthetic target DNA were added per well (for example, see Fig. 4). Even with 1 ×
10^{11} molecules of synthetic target per well, the cross-reactivity was typically <1%, except with the mutation 3905insT probes (Table 2). The cross-reactivity of a certain probe with the PCR product could be measured only if a homozygote mutant sample were available. For all the probes measured, the cross-reactivity with PCR products was comparable with the cross-reactivity observed with the same concentration of synthetic target (data not shown). For example, the mutant ΔF508 probe had a cross-reactivity of 0.9–1.0% with the amplified wild-type DNA, which was directly proportional to the cross-reactivity with the same amount of synthetic target. Because the sequence of the mutant 3905insT differs from the wild-type sequence only by one additional T to T₆ stretch, the 3905insT probes were the most cross-reactive ones (Table 2). Regardless of this we have succeeded in separating the wild-type and mutant alleles of 3905insT in a tolerable range by using very short probes.

Fourteen allele-specific detection probes labeled with lanthanide chelates were used in this hybridization assay. To perform all the hybridizations simultaneously, the hybridization conditions were optimized to be the most satisfying compromise for all the probes. The temperature used in the hybridizations (RT) was chosen according to the temperature profiles of each probe. The system was tolerant to temperature changes between 18 and 25 °C whereas 30 °C was crucial for some probes (Table 3). Therefore, in some laboratories, temperature control would be required.

The signal-to-noise ratios and hybridization efficiencies of each probe at different probe concentrations were determined (e.g., see Fig. 2). The hybridization efficiency proved to be highly sequence-specific as the maximal efficiency varied from 2.5 to 38% (Table 2). The maximum was typically obtained with probe concentrations <1 ng/well, but because the concentrations indicating the highest signal-to-noise ratios were chosen to be used in hybridizations (Table 2), the efficiency of all the probes was lowered.

The detection limit of a probe was defined as the lowest detectable amount of synthetic target DNA, i.e., mean background signal + 3SD. The detection limit of the probe was determined mainly by the lanthanide used in labeling, as observed previously [26]. The most sensitive probes were Eu- and Tb-labeled, while the least sensitive ones were Sm-labeled, varying from 2 × 10⁷ to 3 × 10⁸ (Table 2). Differences in the detection limits of the probes labeled with the same lanthanide were caused predominantly by nonspecific binding but also by the hybridization efficiency. The hybridization signal was linear up to 3 × 10^{10}–1 × 10^{11} molecules of target DNA per well, i.e., at least 3 orders of magnitude (e.g., see Fig. 4). The typical signals obtained in PCR product hybridization corresponded to 3 × 10^{10}–1 × 10^{11} molecules of synthetic target DNA per well. Consequently, in all cases the detection limit was sufficient for analyzing amplified samples.

The assay was verified with purified DNA samples having known mutations. Also, 80 blood spot specimens from CF patients, their parents, and their siblings were analyzed. The hybridization signals of blood spot specimens were typically lower than the signals of purified DNA samples (Table 4), suggesting that the starting concentration in amplification of blood specimens was lower. The blood spot specimens were amplified after pretreatment and also directly from blood spots. With pretreatment, higher concentrations of amplification products were achieved, but the ease of direct amplification makes the method preferable if sufficient amounts of the amplification products can be obtained. In this study, the cutoff was set to signal-to-noise ratio 10. All the analyzed samples confirmed by sequencing were diagnosed correctly (Table 4 and Fig. 5). The cutoff values should be revised in a more extensive study, and they could be defined specifically for each probe. By validating the assay with a large amount of samples, the hybridizations could be further simplified when duplicate hybridizations could be reduced to single hybridizations.

The triple-label hybridization assay using time-resolved fluorometry has proven to be a very convenient method for detection of multiple mutations. In this study, we developed a hybridization assay for seven frequent CFTR mutations. The method is rapid and straightforward. The time and reagent volumes used per hybridization are greatly diminished as three hybridizations can be performed simultaneously in one well. This hybridization assay was set up to function at RT, possibly automating the procedure. In addition, data are collected in numerical form, making the interpretation easy because the data can be processed by a computer, and the cutoff values can be set to a value that excludes cross-reactions. Modifying the pattern of mutations to detect according to the specific requirements in a certain population would be uncomplicated.

This study was supported by the Finnish Graduate School of Bioorganic Chemistry. We thank Teija Ristelä for reviewing the manuscript.

References


