Detection of specific DNA sequences by fluorescence amplification: A color complementation assay

(polymerase chain reaction/fluorescein/rhodamine/fluorometry/color complementation)

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ABSTRACT We have developed a color complementation assay that allows rapid screening of specific genomic DNA sequences. It is based on the simultaneous amplification of two or more DNA segments with fluorescent oligonucleotide primers such that the generation of a color, or combination of colors, can be visualized for disease diagnosis. Color complementation assay obviates the need for gel electrophoresis and has been applied to the detection of a large and small gene deletion, a chromosomal translocation, an infectious agent, and a single-base substitution. DNA amplification with fluorescent oligonucleotide primers has also been used to multiplex and discriminate five different amplified DNA loci simultaneously. Each primer set is conjugated to a different dye, and the fluorescence of each dye respectively to its amplified DNA locus is scored on a fluorometer. This method is valuable for DNA diagnostics of genetic, acquired, and infectious diseases, as well as in DNA forensics. It also lends itself to complete automation.

The identification of specific DNA sequences in humans has not only contributed to the fundamental understanding of the etiology of diseases but also has practical application in DNA diagnostics. In genetic diseases, for example, the more than 70 naturally occurring mutations found in the human β-globin gene (1) have helped to identify DNA regions involved in the control of expression, transcription, and RNA processing of this gene (2). Their identification has also allowed implementation of prenatal diagnosis from fetally derived DNA (3). In genetic diseases in which the mutations have not been elucidated but the gene of interest has been mapped or isolated, linkage analysis is used. Hemophilia A (4), cystic fibrosis (5), Huntington disease (6), polycystic kidney disease (7), and muscular dystrophy (8) are examples of restriction fragment length polymorphisms that have been informative in carrier detection and prenatal diagnosis. In malignant diseases, the point mutations affecting oncogenes in some cancers are amenable to DNA diagnosis, as are chromosome translocations in lymphomas and leukemias (9). DNA analysis has also permitted the rapid identification of etiologic agents in infectious disease (10).

Advances in molecular biology techniques have contributed to the continued improvement of the methodology for detecting DNA sequence variation. Southern blotting has been used to identify large gene deletions (11), chromosomal translocations (9), and restriction fragment length polymorphisms (12). Single-base substitutions not recognized by restriction enzymes do not resolve on Southern blots and are commonly detected by allele-specific oligonucleotide probes (13), RNase cleavage (14), denaturing gradient gel electrophoresis (15), or chemical cleavage (16).

A major advancement in technology has been the ability to amplify specific nucleic acid sequences by the polymerase chain reaction (PCR) (17, 18). When coupled to a thermostable DNA polymerase, PCR has been used to detect gene deletions and point mutations (19, 20) as well as chromosomal translocations (21) and to identify infectious agents (9). However, following PCR, further analysis such as DNA sequencing (22, 23), gel electrophoresis (24), or oligonucleotide hybridization with radioactive (25) or biotin-labeled probes (26) is often required. More recently, a ligase-mediated amplification reaction for allele-specific detection has been used (27). These detection schemes involve manipulations that may be difficult to implement in laboratories that test a large number of samples.

We have devised a quick, easy, and nonradioactive PCR-based color complementation assay that detects infectious agents and a variety of mutations in human DNA. It is based on the simultaneous amplification of two or more DNA segments with fluorescent oligonucleotide probes and visualization of the resulting amplified color by UV light irradiation.

MATERIALS AND METHODS

Source and Isolation of Genomic DNA. Genomic DNA was isolated by standard procedures (28) from the leukocytes of an individual with hydrops fetalis due to complete deletion of the α-globin genes, from β-thalassemia patients heterozygous and homozygous for the 4-base-pair (bp) deletion at codons 41/42 of the β-globin gene, from β-thalassemia patients homozygous and homozygous for the cytidine to thymidine nucleotide substitution at intervening sequence 1 (IVS1) position 110 of the β-globin gene, and from the follicular lymphoma cell line FL218 carrying the 14;18 translocation. DNA from a paraffin-embedded lung tissue specimen infected with cytomegalovirus (CMV) was extracted as reported (29).

Conjugation of Fluorescent Dyes to Oligonucleotide Primers. The oligonucleotides with a primary amino group (Aminolink-2, Applied Biosystems) attached to the 5' end were synthesized on an Applied Biosystems 380B DNA synthesizer. Three optical density units of the crude oligonucleotide mixture were incubated at room temperature with 6 μl of one of the following NHS ester dyes (83.3 μg/ml) in dimethyl sulfoxide: 4′,5′-dichloro-2′,7′-dimethoxy-6-carboxyfluorescein (JOE), 5′-carboxyfluorescein (FAM), 6-carboxytetramethylrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX) (all from Applied Biosystems), or 7-amino-4-methylcoumarin-3-acetic acid (COUM, Molecular Probes). JOE and FAM both fluoresce with yellowish green and orange fluorescence, respectively.

Abbreviations: PCR, polymerase chain reaction; CMV, cytomegalovirus; IVS1, intervening sequence 1; JOE, 4′,5′-dichloro-2′,7′-dimethoxy-6-carboxyfluorescein; FAM, 5′-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; ROX, 6-carboxy-X-rhodamine; COUM, 7-amino-4-methylcoumarin-3-acetic acid.

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green, TAMRA and ROX fluoresce red, and COUM fluoresces blue. The conjugation reaction was carried out in 0.22 M NaHCO₃/Na₂CO₃ at pH 9.0 for 3 hr. The unconjugated dye was separated from the oligonucleotide mixture by Sephadex gel-filtration (G-25) chromatography in 0.1 M triethylamine acetate. The dye-labeled oligonucleotides were then purified from the nonconjugated oligonucleotide by HPLC on an Aquapore 300C-8 column in two consecutive gradients of acetonitrile in 0.1 M triethylamine acetate. The first gradient consisted of 8-20% acetonitrile for 24 min; the second consisted of 20-40% acetonitrile for 10 min. The recovered fluorescent oligonucleotide probes were dried down and resuspended in sterile distilled water.

Amplification Reactions, Gel Electrophoresis, and Removal of Unextended Primers. Thirty picomoles of each primer was mixed with 1 µg of genomic DNA in a 100-µl reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.1), 1.5 mM MgCl₂, and dATP, dGTP, dCTP, and TTP each at 125 µM. The mixture was incubated at 95°C for 5 min to separate the DNA strands and cooled on ice for 5 min; 5 units of *Thermus aquaticus* DNA polymerase was added, and the reaction mixture was overlaid with 35 µl of mineral oil. Thirty amplification cycles were performed on a Perkin-Elmer/Cetus thermal cycler at 95°C for 30 sec, 50°C for 30 sec, and 68°C for 30 sec for all amplification reactions except the 14;18 translocation, for which the annealing temperature was 58°C. Following PCR, two methods were used to visualize the amplified DNA. (i) Aliquots (10–15 µl) of each amplification mixture were loaded on a 0.1 × 10 × 0.1 cm 8% or 10% polyacrylamide gel in 90 mM Tris/64.6 mM boric acid, pH 8.0. The gels were visualized on a long wavelength (~300 nm) UV transilluminator. (ii) To remove the unincorporated primers, the reaction mixture was diluted to 2.5 ml with 10 mM Tris (pH 8.0) and loaded onto a Centricon-100 microfiltration tube (Amicon) and centrifuged at 5000 rpm for 10 min in a fixed-angle rotor (Sorvall SS35). The amplified DNA retained on top of the filter (45–50 µl) was recovered, transferred to conical tubes, and visualized on the transilluminator. Gels or tubes containing the amplified DNA were photographed with a Polaroid MP-4 Land camera using Polaroid color film (type 668; 80 ASA). Each film was exposed twice at f4.5 for 30 sec to 1 min, first with a Wratten gelatin filter no. 16 and then with Wratten gelatin filter no. 21A.

Fluorescent Multiplex PCR. To determine whether we could detect five different fluorescent-amplicated DNA segments simultaneously, we used a mixture of five different oligonucleotide probes, each labeled with a different fluorophore to test DNAs obtained from an individual with hydrops fetalis and one patient each heterozygous or homozygous for a 4-bp deletion at codons 41/42 or heterozygous or homozygous for the cytidine to thymidine mutation at IVS1 position 110. The five probes were homologous to the mutant and normal sequence at codons 41/42, the mutant and normal sequence at IVS1 position 110, and the α-globin gene. The probes were labeled with FAM, ROX, JOE, TAMRA, and COUM, respectively. Following DNA amplification and removal of the unincorporated primers by two rounds of Centricon-100 centrifugation, the fluorescence of each dye was determined on a Perkin-Elmer LS-5 fluorometer. COUM, FAM, JOE, TAMRA, and ROX were excited at 350, 488, 520, 550, and 585 nm, respectively, and the fluorescence emitted at 450, 520, 545, 580, and 605 nm, respectively, was determined. The background fluorescence from a reaction containing all components except the DNA template was subtracted from the sample readings.

**RESULTS**

Color Complementation Assay. When two DNA segments (A and B) are amplified simultaneously, the amplified products can be distinguished from each other if the primers are labeled with different fluorophores—for example, fluorescein (green) for A and rhodamine (red) for B (Fig. 1). If only one of the two segments is amplified, the amplified product will emit either a green or a red color. If both are amplified, the amplified products may be separated by electrophoresis, and if one segment is smaller than the other (A < B), the green and red bands can be resolved. If the segments are equal in length, a single yellow band will be seen as a result of the complementation of the green and red fluorescence. Instead of using gel electrophoresis, the color of the amplified products can also be visualized directly after removal of the unincorporated primers. In this instance, when both segments are amplified, the products emit a yellow color, whether or not they are equal in length. This amplification strategy can be used to detect gene deletions, chromosome translocations, and infectious agents, as illustrated by the three examples shown below.

To detect point mutations and small deletions, three primers are used in the amplification reaction (Fig. 2). Two of the primers, which are located at the site of the mutation, differ from each other only at the position of the mutation. The one corresponding to the normal sequence is labeled with rhodamine (red) and the other, corresponding to the mutant sequence, is labeled with fluorescein (green). The third primer, which is complementary to the opposite strand, is unlabeled. During PCR the completely homologous primer is utilized much more efficiently than the mutant one. Hence, the red color product is emitted by the normal DNA, green is emitted by the homozygous mutant DNA, and yellow is emitted by the heterozygous DNA because of the color complementation of the green and red fluorescence. This assay is illustrated with two β-thalassemia mutations.

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Gene Deletion in Hydrops Fetalis. The α-globin-specific primers, which amplify a 136-bp DNA segment, are labeled with ROX, while the β-globin specific primers, which amplify a 110-bp segment, are labeled with FAM. In normal DNA, both the red (ROX) and green (FAM) amplified bands can be seen after gel electrophoresis, while in hydrops DNA only the green amplified band is seen (Fig. 3). After removing most of the unincorporated primers by centrifugation (two right lanes), the color of the amplified DNA can be visualized directly (bottom). Thus, a yellow color indicates a normal DNA sample, while a green color is diagnostic of α-globin gene deletion.

Chromosome Translocation. We used the 14;18 translocation in follicular lymphoma as an example (30). One pair of primers, positioned across the breakpoint of the translocation, amplify a 185-bp DNA fragment and are labeled with ROX. As an internal control, primers labeled with FAM amplify a 150-bp α-globin gene fragment. Whereas only a green band representing α-globin is seen in normal DNA, the presence of the 14;18 translocation in DNA from follicular lymphoma gives rise to a red band as well (Fig. 4). If the mixture is visualized directly after centrifugation, a green color would indicate the absence of the 14;18 translocation, whereas a yellow color denotes its presence.

Infectious Agents. We used the CMV as an example. The CMV primers were labeled with ROX, and the control β-globin primer was labeled with FAM. In infected samples, a 100-bp red band amplified from the CMV sequences is readily discernible in addition to the green β-globin control band (110 bp) (Fig. 5). In the tube assay, the infected sample emits a yellow color, while an uninfected one fluoresces green.

β-Thalassemia Due to a 4-bp Deletion or a Single Base Pair Substitution. A 4-bp deletion at codons 41/42 of the β-globin gene is the most common mutation responsible for β-thalassemia in the Chinese (31). Of the two primers homologous to this region of the DNA, the one corresponding to the normal sequence was labeled with ROX, and the mutant primer was labeled with FAM. In normal DNA, a red band was seen; in the homozygous DNA a green band was seen (Fig. 6). Because the two amplified products differ by 4 bp (108 vs. 104 bp), we were able to resolve the red and green bands by electrophoresis. In the tube assay, the normal, heterozygous, and homozygous states could be distinguished by their red, yellow, and green fluorescence, respectively.

A single nucleotide mutation can also be discriminated with this assay. Fig. 7 illustrates the detection of a cytidine to thymidine mutation at IVS1/position 110, the most common

![Fig. 2. Strategy for color complementation assay detection of point mutations. A cytidine to thymidine mutation is illustrated in the example. Two allele-specific primers corresponding to this region are labeled with red dye (corresponding to the wild-type allele) or green dye (mutant allele). The primer amplifying the opposite strand is unlabeled. After PCR and removal of unincorporated primers, the amplified products for normal, heterozygous, and homozygous DNA are red, yellow, and green, respectively.](image)

![Fig. 3. Detection of a large deletion in genomic DNA. The pair of primers that amplify the 136-bp α-globin gene were labeled at their 5' end with ROX and have the sequences 5'-TACTGTAGATACCCGTGACA-3' and 5'-ATCATGATGGAAACATAGTAAT-3'. The sequences of the primer pair amplifying the internal control (110 bp, β-globin) were 5'-CAGAGGGTTCTTT-GAGTCCT-3' and 5'-GCCATCATAAGGCACCG-3', both labeled with FAM. Shown on the gel is a fractionation of an aliquot from each amplification reaction before and after removal of the unextended primers by Centricon-100 microfiltration. The tubes show the color of each amplified reaction after filtration. Amplified normal DNA is yellow by color complementation, whereas hydrops fetalis DNA is green.](image)

![Fig. 4. Detection of the t(14;18) chromosomal translocation. The sequences of the primer pair used to amplify the breakpoint were 5'-CTTTTGGAGATTTGCTTACGT-3' and 5'-ACCTGTAGGAGACGGTGACCAG-3', both labeled with ROX. The pair of primers used to amplify the internal control were 5'-TGGACCCCTACATCATGATACCCGTGACA-3' and 5'-ATCATGATGGAAACATAGTAAT-3', both labeled with FAM. Gel electrophoresis: The amplified α-globin internal control DNA is 150 bp long and the amplified breakpoint segment between chromosomes 14 and 18 is 185 bp long. Color complementation in the tubes: Normal DNA is green; t(14;18) DNA is yellow. Note that the double bands produced as artifacts of electrophoresis did not interfere with the interpretation of the results.](image)
Fig. 5. Detection of CMV. The sequences of the primer pair used to amplify the viral DNA were 5'-TTGCTGTGTCGCCGG-GACCC-3' and 5'-GCACGGGCAAAGAAGACCG-3', both labeled with ROX. The internal control was the same FAM-labeled primers as in Fig. 3. Gel electrophoresis: The 100-bp amplified CMV DNA and the 110-bp internal control β-globin DNA are shown on the gel. Color complementation: Normal DNA fluoresces green and CMV-infected DNA is yellow.

β-thalassemia lesion found in people of Mediterranean ancestry (32). The red, yellow, and green color from normal, heterozygous, and homozygous samples, respectively, can be readily discriminated, both by electrophoresis and by tube assay.

Simultaneous Determination of Five Different PCR Products. Table 1 illustrates the results of simultaneously amplifying with five pairs of primers, each labeled with a different fluorescent dye. DNA from individuals with five different known diagnoses was tested. The relative fluorescence values obtained on the fluorometer are not quantitative, but a value of less than 1.0 was considered as negative and more than 1.0 as positive for hybridization. Thus, DNA from an individual homozygous for the mutation at codons 41/42 failed to hybridize to the normal probe at this position and to the mutant probe at IVS1 position 110 because the latter mutation is not present. But as expected, the DNA hybridized to the mutant probe at codons 41/42, to the normal probe at IVS1 position 110, and to the α-globin probe. Similarly, the appropriate genotype at these loci could also be correctly diagnosed in the DNA from the other four patients.

![c](image)

**Fig. 6.** Detection of a 4-bp deletion in the β-globin gene. The two allele-specific primers—5'-CAGAGGTCTTCTGAGTCT-C-3' (normal) and 5'-CAGAGGTCTTCTGAGTCT-C-3' (mutant)—were labeled with ROX and FAM, respectively. The sequence of the downstream primer was 5'-GCATCACAAAGGCCAC-3', and this primer was not dye-conjugated. Gel electrophoresis: The rhodamine wild-type and fluorescein mutant allele-specific oligonucleotides amplify a 108- and 104-bp segment, respectively. A normal individual shows only the 108-bp red band, a heterozygous shows both the 108-bp red and 104-bp green bands, and the homozygous mutant individual shows only the green band. Color complementation: Normal DNA is red, heterozygous DNA is yellow, and homozygous mutant DNA is green.

![c](image)

**Fig. 7.** Detection of a single-nucleotide substitution in IVS1 position 110 of the β-globin gene. The two allele specific primers—5'-CTGCTATTGCTATT-TTT-3' (normal) and 5'-CTGCTATT-GCTATT-TTT-3' (mutant)—were conjugated to ROX and FAM, respectively. The downstream primer, 5'-GCCATCAC-AAAGGCCAC-3', was not labeled. Gel electrophoresis: Amplification results in a 165-bp DNA segment. The normal and homozygous mutant DNA produce a red and a green band, respectively. A heterozygous individual shows a single yellow band as a result of the complementation of red and green fluorescence. Color complementation: Normal DNA is red, heterozygous DNA is yellow, and homozygous mutant DNA is green.

Table 1. Fluorometric detection of multiple DNA loci by color PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>4-bp deletion (FAM)</th>
<th>IVS1 position 110 (ROX)</th>
<th>Mutant (JOE)</th>
<th>Normal (TAMRA)</th>
<th>α-Globin (COUM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>4.6</td>
<td>0.7</td>
<td>0.4</td>
<td>1.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Homozygous 4 bp</td>
<td>5.1</td>
<td>7.5</td>
<td>0.8</td>
<td>2.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Heterozygous 4 bp</td>
<td>0.4</td>
<td>13.9</td>
<td>0.8</td>
<td>2.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Hydrops fetalis</td>
<td>0.8</td>
<td>13.8</td>
<td>1.9</td>
<td>1.8</td>
<td>9.3</td>
</tr>
<tr>
<td>Heterozygous 110</td>
<td>0.8</td>
<td>11.5</td>
<td>4.8</td>
<td>0.2</td>
<td>7.7</td>
</tr>
<tr>
<td>Homozygous 110</td>
<td>0.8</td>
<td>13.8</td>
<td>1.9</td>
<td>1.8</td>
<td>9.3</td>
</tr>
</tbody>
</table>

The fluorescence values were determined by fluorometry at the excitation/emission wavelengths characteristic of each dye. The italic foldback values indicate positive amplifications (>1.0), all of which correspond to the expected results. The primers to amplify the α-globin gene were the same as in Fig. 3, except that they were conjugated to COUM. The dye allele-specific and downstream primers for codons 41/42 of the β-globin gene were the same as those in Fig. 6. The normal and mutant allele-specific primers for the IVS1 position 110 of the β-globin gene were identical to those in Fig. 7, except that they were conjugated to TAMRA and JOE, respectively. The downstream primer for this reaction was 5'-AGAACCTC-TGGGTCCAAGG-3' (unlabeled).
DISCUSSION

We describe a nonradioactive, fluorescence-based detection system for identifying specific DNA sequences amplified by PCR. The main advantage of this system is that it obviates the complicated procedures often needed to detect the results of the amplification reaction, and it allows genotyping of individuals by direct visualization of color following gel electrophoresis or centrifugation. Procedures such as restriction endonuclease digestion (19, 24), oligonucleotide (25) or reverse oligonucleotide hybridization (33), ligase amplification (27), and DNA sequencing (22, 23) are therefore not necessary. When the diagnosis rests upon the presence or absence of a sequence, as in the case of gene deletions, chromosome translocations, and infectious diseases, only two different color primers are needed, one for the test and one as an internal control for the PCR reaction. When detecting point mutations, two different allele-specific colored primers situated at the location corresponding to the normal and mutant sequence will distinguish the wild type from the mutant, as a completely homologous primer is utilized an order of magnitude more efficiently than one with even a single base pair mismatch (F.F.C., unpublished observations).

In some instances, it may be desirable to perform multiple DNA amplifications on one DNA sample. Examples of such conditions are (i) genetic diseases caused by multiple mutations (34–36), (ii) infectious diseases in which etiologic agents are suspected, and (iii) HLA typing in which multiple loci determinations are necessary (37). Either several amplifications with different primers can be analyzed separately, or simultaneous multiplex amplification with different fluorophore-labeled primers can be performed. We have shown that up to five different DNA amplification products can be determined (Table 1). By choosing the appropriate fluorophores, it may be possible to examine simultaneously even more loci.

An important advantage of the color complementation assay is its adaptability to automation, which is an important consideration when a large number of tests are performed. Two different approaches to automation are possible. By incorporating an appropriate ligand such as biotin to one PCR primer and a fluorophore tag to the other primer, the amplified DNA segments thus carry at each 5′ end a biotin or a fluorophore molecule. The amplified DNA could be separated from the unincorporated fluorescent primers by using streptavidin magnetic beads. The color of the amplified DNA would then be determined by fluorometry through a fiber optic bundle. Alternatively, the PCR products could be separated by electrophoresis, and the color of the bands could be analyzed by laser scanning, as is done for DNA sequencing.

Note Added in Proof. This method has also been used successfully to detect the most common mutation in cystic fibrosis due to the 3-bp deletion (F.F.C., unpublished observations).

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