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Fluorescence energy transfer detection as a homogeneous DNA diagnostic method

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ABSTRACT **A homogeneous DNA diagnostic assay based on template-directed primer extension detected by fluorescence resonance energy transfer, named template-directed dye-terminator incorporation (TDI) assay, has been developed for mutation detection and high throughput genome analysis. Here, we report the successful application of the TDI assay to detect mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, the human leukocyte antigen H (HLA-H) gene, and the receptor tyrosin kinase (RET) protooncogene that are associated with cystic fibrosis, hemochromatosis, and multiple endocrine neoplasia, type 2, respectively. Starting with total human DNA, the samples are amplified by the PCR followed by enzymatic degradation of excess primers and deoxyribonucleoside triphosphates before the primer extension reaction is performed. All these standardized steps are performed in the same tube, and the fluorescence changes are monitored in real time, making it a useful clinical DNA diagnostic method.**

Disease-causing mutations have been identified in an increasing number of genes in recent years using molecular genetic techniques. The rate of discovery of "disease genes" is accelerating rapidly as a result of the progress of the Human Genome Project. As these newly identified genes are analyzed, it is clear that except in a few genetic diseases where one mutation accounts for the majority of the cases, multiple mutant alleles are involved in each disease and that mutations are often family- or population-specific. For example, over 100 unique, disease-causing mutations have been found in the *BRCA1* breast cancer susceptibility gene (1). Among the *BRCA1* mutations, two (185delAG and 5382 insC) are found almost exclusively in Ashkenazi Jewish patients (2, 3). For each new patient of a genetic disorder, identifying the mutation in the candidate gene requires scanning a substantial portion of the gene using laborious techniques such as DNA sequencing. Once the mutation is identified, however, other family members at risk can be screened for the specific disease-causing mutation alone to assess disease risk. Because the majority of mutations in genetic disorders are due to single base pair changes or small insertions or deletions, it is highly desirable to develop a DNA diagnostic test that is highly sensitive, specific, cost-effective, and easy to carry out for each mutation screened in the clinical laboratory.

We have developed a simple, homogeneous DNA diagnostic test using fluorescence resonance energy transfer (FRET) as the basis for detecting single base pair changes in a template-directed primer extension reaction (4). Since the publication of original method, the template-directed dye-terminator incorporation (TDI) assay has been substantially improved to a one-tube, homogeneous assay testing for both the normal and mutant alleles that is accomplished in four steps (Fig. 1). The DNA fragment containing the mutation site is first amplified from genomic DNA by PCR (5). Following the PCR, excess primers and deoxynucleoside triphosphates (dNTPs) are degraded by the addition of *Escherichia coli* exonuclease I and shrimp alkaline phosphatase (6). The enzymes are then heat-inactivated before the primer extension reaction is performed. In the primer extension step the incorporation of normal or mutant chain terminating dideoxynucleoside triphosphates (ddNTPs) is template specific, thus one can determine mutational status by monitoring which dye-labeled ddNTP is incorporated at the mutation site (4).

Fluorescence resonance energy transfer is observed when two fluorescent dyes are in close proximity and one fluorophore's emission overlaps the other's excitation spectrum (7). When the donor dye is excited, its specific emission intensity decreases (quenched) whereas the specific emission intensity of the acceptor dye increases. Changes in fluorescence intensities of the donor and the acceptor can therefore be used as an index for the distances between the two fluorescent dyes. Although the efficiency of FRET is very sensitive to the distance between the donor and acceptor, with energy transfer dropping dramatically as the distance between them increases, the hydrophobic interaction between the organic dye molecules in free solution makes it possible to observe FRET even when the donor and acceptor are placed 20 bases apart on an oligonucleotide (4, 8). The ability to detect intramolecular FRET against the background of intermolecular FRET provides a novel and unique detection system that requires no separation or purification of the product in the TDI assay. The changes in fluorescence intensities during the primer extension step can be monitored in real time by a fluorescence spectrophotometer connected to a thermal cycler or at its end-point by means of a fluorescence plate reader.

We report here the successful application of the TDI assay to test for the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (9) in 38 individuals from families with cystic fibrosis, the C282Y mutation in the human leukocyte antigen H (HLA-H) gene (10) in 49 individuals of known mutational status for hemochromatosis, and for the presence of 18 different mutations in the receptor tyrosin kinase (RET) protooncogene (refs. 11 and 12; H. Donis-Keller, personal communication) in 22 families (95 individuals) with multiple endocrine neoplasia type 2 (MEN2) or familial medullary thyroid carcinoma (FMTC). The ΔF508 mutation in the CFTR gene is a 3-base deletion found in >70% of North American and Western European Caucasian cystic

Abbreviations: FRET, fluorescence resonance energy transfer; TDI, template-directed dye-terminator incorporation; ROX, 6-carboxy-X-rhodamine; TAMRA, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine; CFTR, cystic fibrosis transmembrane conductance regulator; HLA, human leukocyte antigen; MEN, multiple endocrine neoplasia; FMTC, familial medullary thyroid carcinoma; RFLP, restriction fragment length polymorphism; RET, receptor tyrosin kinase.

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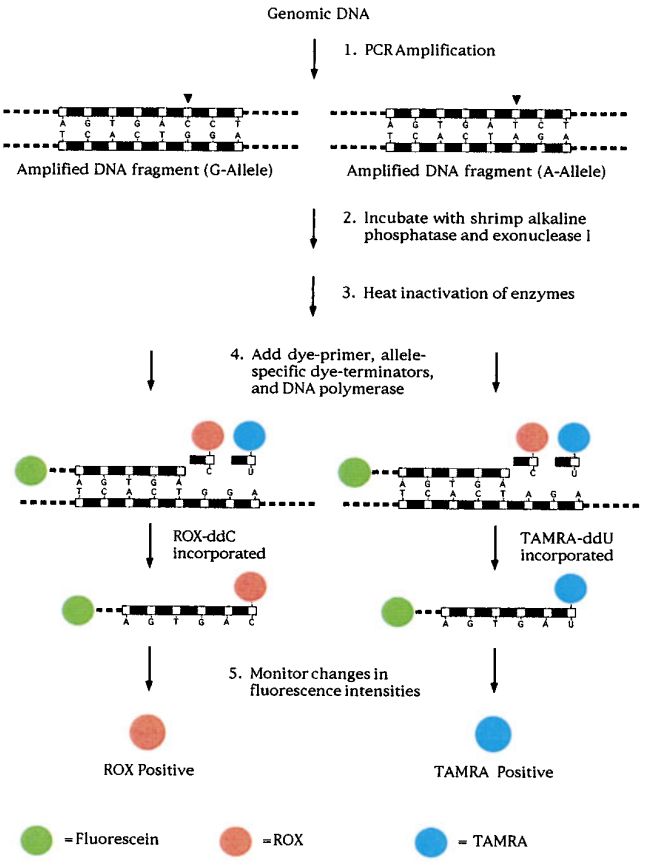

FIG. 1. The TDI assay.

fibrosis patients (9), and the C282Y mutation in the HLA-H gene is a single base pair mutation found on >85% of the chromosomes in hemochromatosis patients (10). Missense mutations in five conserved cysteine codons of exons 10 and 11 of the RET protooncogene are associated with the autosomal dominant inheritance of the cancer family syndromes, MEN2A and FMTC (refs. 11–13; H. Donis-Keller, personal communication). A single methionine codon in exon 16 is associated with the clinically distinct entity, MEN2B (14, 15). The existence of 18 different mutations in the moderate number of families in the Washington University collection poses a challenge to the DNA diagnostic laboratory in testing each affected family for its specific mutation. Currently, the base changes are detected by PCR plus restriction fragment length polymorphism (PCR-RFLP) or direct DNA sequencing of a PCR product which spans the exon where the mutation resides (16). To simplify this task, TDI assays were developed for all 18 mutations identified in the MEN2 families at Washington University (ref. 12; H. Donis-Keller, personal communication).

MATERIALS AND METHODS

Oligonucleotides. PCR and TDI primers were custom synthesized by Genset (La Jolla, CA). The 5′ fluorescein-labeled TDI primers were purified by reverse-phase HPLC liquid chromatography by Genset. The PCR primer sequences (5′–3′) were as follows: CF508p1, GTGCATAGCAGAGTACCTGAAACAGGAAGTA; CF508p2, TGATCCATTCACAGTAGCTTACCCATAGAGG; HLAHp1, TGGCAAGGGTAAACAGATCC; HLAHp2, CTCAGGCACTCCTCTCAACC. The MEN mutations are found in three exons (exons 10, 11, and 16) that were amplified by three sets of PCR primers: MEN10p1, GCGCCCCAGGAGGCTGAGTG; MEN10p2, CGTGGTGGTCCCGGCCGCC; MEN11p1, CCTCTGCGGTGCCAAGCCTC; MEN11p2, CACCGGAAGAGGAGTAGCTG; MEN16p1, AGAGAGTTAGAGTAACTTCAATGTC; MEN16p2, CTACATGTATAAGGGTGTTT. The TDI primer sequences were as follows: CF508, 5′-F-CTGGCACCATTAAAGAAAATATCAT; HC282Y, 5′-F-GGAGAGCAGAGATATACGT; MENC634F, 5′-F-CCACTGTGCGACGAGCTGT.

Enzymes and Nucleotides. AmpliTaq DNA polymerase was purchased from Perkin–Elmer. Shrimp alkaline phosphatase and *E. coli* exonuclease I were obtained from Amersham. Klentaq1-FY was obtained from the laboratories of Wayne Barnes (Washington University). [Note that AmpliTaq, FS (Perkin-Elmer) and ThermoSequenase (Amersham) gave the same results in the TDI assay.] dNTPs and ddNTPs were purchased from Pharmacia. ddNTPs labeled with 6-carboxy-X-rhodamine (ROX-ddA, ROX-ddC, ROX-ddG, and ROX-ddU) or with *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA-ddA, etc.) were obtained from NEN.

PCR Conditions. Amplification was done in 0.2 ml MicroAmp Optical tubes (Perkin–Elmer) in a GeneAmp 9600 thermal cycler (Perkin–Elmer). Human genomic DNA (20 ng) from each individual was amplified in a 10-μl reaction mixture containing 50 mM Tris·HCl (pH 9.0), 50 mM KCl, 5 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of each PCR primer, and 1 unit of AmpliTaq DNA polymerase. Thermal cycling conditions to amplify the DNA fragment containing the cystic fibrosis mutation were as follows: initial denaturation at 94°C for 180 s followed by 10 cycles at 94°C for 10 s, ramping to 68°C over 90 s, 68°C for 30 s, followed by 30 cycles at 94°C for 10 s, 62°C for 30 s. For the hemochromatosis C282Y mutation, the annealing/extension temperature was 68°C for the first 10 cycles and 62°C for the last 30 cycles. For the MEN2 (C634F) mutation (exon 11), the annealing/extension temperature was 60°C for the first 10 cycles and 53°C for the last 30 cycles. For MEN2 mutations found in exons 10 and 16, the thermal cycling conditions were the same as those for MEN2 exon 11 except that the annealing/extension temperatures were 72°C and 56°C for the first 10 cycles, and 68°C and 50°C for the last 30 cycles, respectively.

Primer and dNTP Degradation Conditions. At the end of the PCR assay, 10 μl of an enzymatic mixture containing shrimp alkaline phosphatase (2 units), *E. coli* exonuclease I (1 unit) in shrimp alkaline phosphatase buffer (20 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$) was added to the PCR product. The mixture was incubated at 37°C for 30 min before the enzymes were heat inactivated at 95°C for 15 min. The DNA mixture was kept at 4°C and was used in the TDI assay without further quantitation or characterization.

TDI Assay Conditions. Ten microliters of TDI reaction mixture (50 nM TDI primer/200 nM allele-1-specific ROX-ddNTP/200 nM allele-2-specific TAMRA-ddNTP/1 μM of each of the other two unlabeled ddNTPs in 50 mM Tris·HCl, pH 9.0/50 mM KCl/5 mM NaCl/5 mM $MgCl_2$/8% glycerol/0.1% Triton X-100/0.2 unit Klentaq1-FY) were added to the enzyme-treated PCR product. Optical caps were placed on the reaction tubes before thermal cycling was performed in the Applied Biosystems Prism 7700 Sequence Detector (Perkin–Elmer). Thermal cycling conditions for the TDI assay to test for the cystic fibrosis ΔF508 mutation was done by an initial denaturing step at 95°C for 60 s, followed by 35 cycles at 95°C for 10 s and 50°C for 30 s. For the hemochromatosis and MEN2 (C634F) mutations, the annealing/extension temperature was 60°C.

Genotype Analysis. The fluorescence intensities were acquired during the annealing/extension phase of the primer extension cycles. This was necessary because of the significant reduction in fluorescein fluorescence intensity at high temperatures. The analysis was done using the multicomponent data from the Applied Biosystems 7700 Sequence Detector. The ROX and TAMRA fluorescence intensities of each cycle

were averaged, and the initial rate of change (initial slope) in the average fluorescence intensity for each dye was calculated by linear regression for cycles 2–5. The average intensity of cycle 1 was not used because there was often a slight dip in fluorescence intensity during the first thermal cycle. The genotype of a sample was scored using the following criteria: (*i*) initial slope of <5 fluorescence units/cycle for both alleles = no reaction (sample contained neither alleles or PCR failure); (*ii*) initial slope of <5 units/cycle for one allele and >10 units/cycle for the other allele = homozygous for the allele with significant change; and (*iii*) initial slope of >5 units/cycle for both allele: (*a*) the allele 1/allele 2 ratio is >4 = homozygous for allele 1; (*b*) the allele 1/allele 2 ratio is <0.25 = homozygous for allele 2; and (*c*) the allele 1/allele 2 ratio is between 0.33 and 3 = heterozygote.

RESULTS AND DISCUSSION

In these experiments, genomic DNA samples from individuals previously tested for the cystic fibrosis, hemochromatosis, and MEN mutations were genotyped in a blinded study. Amplified DNA fragments were incubated with a fluorescein-labeled sequencing primer (designed to hybridize to the DNA template adjacent to the mutation site) in the presence of the two allelic ROX-ddNTP or TAMRA-ddNTP terminators and a modified *Taq* DNA polymerase (Klentaq1-FY). If the template contains the base complementary to the dye-terminator in the reaction, the dye-terminator is incorporated and the dye-primer is extended by one base. The mutational status of the DNA sample is determined simply by analyzing the real-time fluorescence intensity profiles of the three dyes in the reaction mixture on a fluorescence thermal cycler.

Fig. 2 shows the real-time fluorescence intensity profiles of four representative samples tested for the ΔF508 (delCTT) mutation in the CFTR gene during linear amplification in the primer extension step of the TDI assay. The fluorescence readings correspond to the emission maxima for each of the three fluorescent species (fluorescein, ROX, and TAMRA) corrected for the interfering fluorescence contributed by the other two species using multicomponent analysis. In Fig. 2*A*, the normal C/C sample incorporates ROX-ddC but not TAMRA-ddU and shows a progressive drop in fluorescein fluorescence and a corresponding rise in ROX fluorescence whereas the TAMRA fluorescence remains unchanged. The homozygous mutant T/T sample in Fig. 2*B* incorporates TAMRA-ddU but not ROX-ddC and shows a drop in fluorescein fluorescence and a corresponding rise in TAMRA fluorescence whereas the ROX fluorescence is unchanged. The heterozygous sample incorporates both ROX-ddC and TAMRA-ddU and shows a drop in fluorescein fluorescence with a rise (albeit less pronounced) in both the ROX and TAMRA fluorescence (Fig. 2*C*). In contrast, the fluorescence intensity profiles of a negative control sample (salmon sperm DNA template, Fig. 2*D*) are unchanged for all three dyes because neither ROX-ddC nor TAMRA-ddU are incorporated. In each case, the rise in ROX and TAMRA fluorescence intensities is the greatest during the first 10 cycles and is largely completed by cycle 15. The rate of change of the ROX and TAMRA fluorescence intensities is a reflection of the template concentration, with the initial slope of change for the

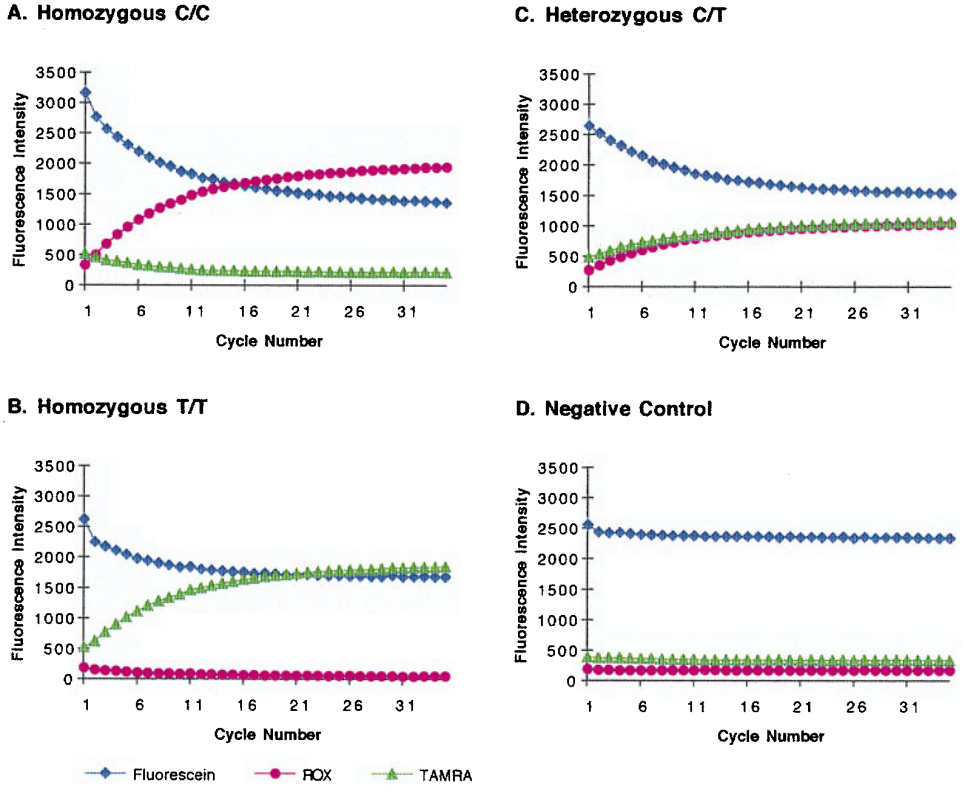

FIG. 2. Real-time fluorescence detection in the TDI assay for the cystic fibrosis ΔF508 mutation. The average fluorescence emissions of the three dye species for each cycle during the primer extension reaction are plotted against the cycle number. Blue diamonds = fluorescein emission, magenta circles = ROX emission, green triangles = TAMRA emission. (*A*) Normal individual showed progressive quenching of fluorescein emission with concomitant enhancement of ROX emission (initial slope = 136) but no significant change in TAMRA emission, signifying that ROX-ddC was the only nucleotide incorporated. (*B*) Affected individual showed quenching of fluorescein and enhancement of TAMRA (initial slope = 118) emission but no significant change in ROX emission, signifying that only TAMRA-ddU was incorporated. (*C*) Carrier showed quenching of fluorescein and enhancement of both ROX and TAMRA emissions (initial slopes of 72 and 51, respectively), signifying that both ROX-ddC and TAMRA-ddU were incorporated. (*D*) No significant change in fluorescence emission was seen for any of the three dyes because neither ROX-ddC nor TAMRA-ddU were incorporated. The slopes in the heterozygote are about half of those for the homozygotes.

heterozygote approximately half of that for the homozygote. By plotting the initial slope of fluorescence intensity changes for ROX and TAMRA, one can assign the mutational status of each test sample with high confidence.

Fig. 3 shows the typical results of such experiments. The fluorescence intensity changes fall into four categories: homozygotes for allele 1, homozygotes for allele 2, heterozygotes, and no reaction. Fig. 3*A* shows the results of the TDI assay for 38 individuals and 4 negative controls (salmon sperm DNA) in a blinded study to test for the ΔF508 cystic fibrosis mutation. Six individuals exhibit a large initial rate of increase of TAMRA fluorescence intensity with only a small initial rate of increase of ROX fluorescence intensity (red squares) and they occupy the upper left corner of the plot. These results represent cystic fibrosis patients who are homozygous for the ΔF508 mutation where only TAMRA-ddU is incorporated. Thirteen individuals have large increases in ROX fluorescence but little increase in TAMRA fluorescence (green triangles) occupying the lower right corner of the plot. These individuals are normal individuals where only ROX-ddC is incorporated. Nineteen individuals have ROX and TAMRA fluorescence increases that fall between the two different homozygous groups (blue diamonds). These individuals are heterozygous carriers of the mutation and they occupy the area in the middle of the plot. The four control DNA samples show no increase in the fluorescence intensity in either direction and occupy the area near the origin, signifying that no products are formed (open magenta circles). A set of criteria has been established to define the four possible outcomes in each genotyping assay. Using this method of scoring, the mutational status of all 38 individuals were determined correctly for the ΔF508 mutation in the CFTR gene. These results were concordant with those obtained using independent assay by PCR or reverse dot-blot hybridization previously performed in the clinical Molecular Diagnostic Laboratory at Washington University.

Results obtained when 49 individuals and 3 negative control samples were tested for the HLA-H C282Y mutation (G to A substitution) associated with hemochromatosis in a similarly blinded study are shown in Fig. 3*B*. ROX-ddG and TAMRA-ddA were used to test for the normal and mutant alleles, respectively. Again, the samples fall into four groups as before. Forty-eight of 49 human DNA samples and all 3 control samples give unequivocal results and are in complete agreement with those determined previously using the oligonucleotide ligation assay (10). One sample is found near the origin (solid magenta circle) with negative slopes for both alleles, representing a failed PCR assay. This particular DNA sample failed to amplify under all PCR conditions tried.

A third blinded study was done for the C634F mutation (G to T substitution) in the RET protooncogene using genomic DNA samples from 29 individuals of known mutational status. In this experiment, TAMRA-ddG and ROX-ddU were used to

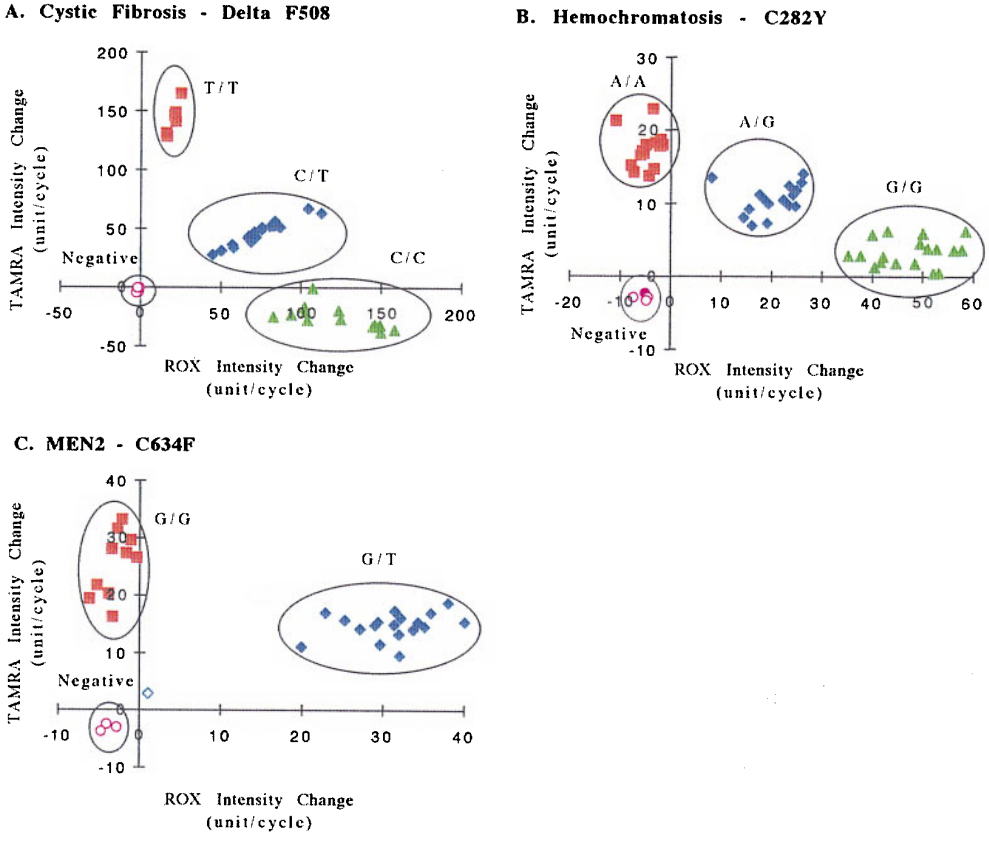

FIG. 3. Detection of known mutations by the TDI assay. The initial slope of change in fluorescence intensity of ROX and TAMRA are plotted against each other. (*A*) Thirty-eight individuals were tested for the cystic fibrosis ΔF508 mutation (with four salmon sperm DNA negative controls), where ROX-ddC and TAMRA-ddU were used in the reaction for the normal and mutant alleles, respectively. The data points clustered into four groups, which represented the normal C/C (green triangles), affected T/T (red squares), and carrier C/T (blue diamonds) populations in addition to the control samples (open magenta circles) where no dye-terminators were incorporated. (*B*) Forty-eight individuals (and 3 negative controls) were tested for the hemochromatosis C282Y mutation, where ROX-ddG and TAMRA-ddA were used for the normal and mutant alleles, respectively. The data points clustered into the normal G/G (green triangles), affected A/A (red squares), carrier G/A (blue diamonds) populations. One individual clusters with the negative controls (open magenta circles) because the PCR assay failed (solid magenta circle). (*C*) Twenty-nine individuals (and 3 negative controls) were tested for the autosomal-dominant MEN C634F mutation, where TAMRA-ddG and ROX-ddU were used for the normal and mutant alleles, respectively. The data points segregated into just the normal G/G (red squares) and affected heterozygous G/T (blue diamonds) groups. One sample had slightly positive slopes of intensity change for both ROX and TAMRA (open blue diamond) due to poor PCR yield whose genotype was deemed indeterminate.

assay for the normal and mutant alleles, respectively. As shown in Fig. 3*C*, only three groups of data points are found. The homozygous mutated allele configuration has not been observed in individuals affected with the autosomal-dominant inherited cancer disorders MEN or FMTC. The genotypes of all but one of the samples determined by the TDI assay are in complete concordance with those determined previously using other methods. The one sample with the indeterminate genotype (open blue diamond near the origin) has slightly positive slopes for both alleles. Agarose gel electrophoresis of the sample showed that the PCR assay produced very little product (data not shown). When the genotyping reaction was repeated, the results showed that this sample was a heterozygote, agreeing with the genotype obtained by PCR-RFLP.

Although the data for this study were generated on a thermal cycler capable of real time fluorescence monitoring, the assay can also be done on a conventional thermal cycler followed by fluorescence detection on a microplate reader. This was accomplished by comparing the end-point fluorescence intensities of the test samples against those of three controls (homozygote for allele 1, homozygote for allele 2, and blank) without the benefit of having the initial fluorescence readings to serve as internal controls. The external controls were necessary to normalize the fluorescence reading and eliminate well-to-well differences. When the fluorescence readings are normalized with the aid of multicomponent analysis based on the external controls, the data points segregate into three distinct groups representing the heterozygous and the two homozygous groups. End-point analysis was performed using the data generated in the three experiments described in Fig. 3 and they produced exactly the same genotypes (data not shown) except for one sample that was indeterminate in addition to the two samples that gave no results due to PCR failure. This sample was scored correctly as a heterozygote by the real-time monitoring approach. End-point assays were also performed to test for the 17 other specific RET mutations in each of the families carrying the mutation in a blinded study. The genotyping results of the 66 samples tested were in complete concordance with those obtained previously by PCR-RFLP or DNA sequencing. The MEN2 mutations tested and probes used are found in Table 1.

The TDI genotyping assay combines the specificity of enzymatic discrimination between the two alleles of a single base pair change or a small insertion/deletion and the sensitivity of fluorescence resonance energy transfer. It is accomplished in four simple steps without the need for separation or purification, and the entire protocol is performed in one reaction vessel. Only two reagent transfer steps are involved once the initial PCR is set up. Furthermore, all four steps can be automated using a robotic work station, a thermal cycler, and a fluorescence spectrophotometer. As a homogeneous genotyping assay, it can be used to process large number of samples in parallel without being limited to a particular format. Even without automation, the entire protocol can be accomplished in 4 h starting with genomic DNA. No false-positive results were encountered in this assay when applied to the 181 genomic DNA samples tested for a total of 20 mutations. Two samples gave indeterminate results (1.1%) due to PCR failure in both analysis formats, and one additional sample gave indeterminate results when end-point analysis was used to determine the genotype.

Two homogeneous genotyping methods based on PCR amplification and FRET detection have recently been described. Both of these methods, the TaqMan (8) and Molecular Beacon (17) assays, rely on allele-specific hybridization to remove the quenching of a fluorescent reporter. Although these two methods are superior to the TDI assay in their ease of reaction set-up because they are one-step reactions, they are much harder to optimize because of the requirement that only the perfectly complementary probe is hybridized to the template during PCR amplification. Furthermore, both methods require two expensive doubly labeled probes that may take several designs to work perfectly. In contrast, the TDI probe is a simple 5′ fluorescein-labeled oligonucleotide similar to those used in dye-primer sequencing reactions. The chemically stable dye-primer requires no optimization and can be purchased from a commercial supplier (100 nmol, enough for 100,000 reactions, for $100) or can be prepared by any laboratory with a DNA synthesizer. The cost of the reagents and plastic ware for the TDI assay (excluding the fixed cost of PCR primer and TDI probe) is around $1 per sample, starting with genomic DNA.

Because the risk for development of hereditary diseases, such as familial breast and ovarian cancer, is associated with specific mutations in the genes involved, a simple, standardized DNA diagnostic test such as the TDI assay will make it possible for any clinical laboratory to design and perform carrier testing within a kindred. Because the TDI assay is easily performed on a large number of DNA samples, it is also suitable for population screening for specific common mutations such as

Table 1. RET protooncogene mutations tested using the TDI assay

Exon/Codon	Base/amino acid change	TDI probe sequence	Annealing temperature, (°C)	Samples tested
10/609	TGC > TAC (C609Y)	F-AAAGCTGGCTATGGCACCT	55	2
10/611	TGC > TGG (C611W)	F-CTATGGCACCTGCAACTG	50	2
10/618	TGC > GGC (C618G)	F-GTCTTCGGGCTCGCAGAAGC	60	4
	TGC > CGC (C618R)	F-GTCTTCGGGCTCGCAGAAGC	60	8
	TGC > AGC (C618S)	F-GTCTTCGGGCTCGCAGAAGC	60	4
	TGC > TTC (C618F)	F-TTCCCTGAGGAGGAGAAGT	55	2
	TGC > TAC (C618Y)	F-TTCCCTGAGGAGGAGAAGT	55	4
10/620	TGC > CGC (C620R)	F-CTGGATGTCTTCGGGCTCGC	65	2
	TGC > TTC (C620F)	F-GAGGAGGAGAAGTGCTTCT	50	2
	TGC > TCC (C620S)	F-GAGGAGGAGAAGTGCTTCT	50	3
	TGC > TAC (C620Y)	F-GAGGAGGAGAAGTGCTTCT	50	6
11/634	TGC > GGC (C634G)	F-GGCTGCGATCACCGTGCGGC	65	5
	TGC > CGC (C634R)	F-GGCTGCGATCACCGTGCGGC	65	8
	TGC > TTC (C634F)	F-CCACTGTGCGACGAGCTGT	60	29
	TGC > TCC (C634S)	F-CCACTGTGCGACGAGCTGT	60	1
	TGC > TAC (C634Y)	F-CCACTGTGCGACGAGCTGT	60	5
	TGC > TGG (C634W)	F-ACTGTGCGACGAGCTGTG	50	7
16/918	ATG > ACG (M918T)	F-TCGGATTCCAGTTAAATGGA	55	1
Total				95

The TDI probes are labeled with fluorescein (F) and the annealing temperature used in the TDI assay are indicated for each probe.

ΔF508 in cystic fibrosis, C282Y in hemochromatosis, or the 185delAG and 5382 insC mutations for breast cancer in Ashkenazi Jewish patients. Moreover, as a high-throughput method, the TDI assay is also useful in population studies with single nucleotide polymorphic markers. As has been discussed recently, dissecting the genetic basis of common diseases such as cardiovascular disease, autoimmune diseases, psychiatric disorders, or susceptibility to cancer will require a genome-wide, dense set of polymorphic markers to be genotyped in thousands of people (18). In contrast to high-throughput genotyping methods based on high-density oligonucleotide arrays (19, 20), the TDI assay offers a high degree of flexibility. Because the homogeneous TDI assay is not limited to any format, new markers can be easily added to the panel as they become available. Therefore, one can choose the optimal set of markers for both the population and the genomic region being studied. As demands for high throughput genotyping is expected to increase dramatically in the areas of diagnostics, forensics, population studies, and agricultural biotechnology, a simple, automated genotyping method will make it possible to perform these studies efficiently and economically. Because DNA diagnostic tests will no doubt be performed more and more by clinical rather than research laboratories, standard protocols such as the TDI assay that require minimal laboratory skills will be crucial to the clinical practice of medicine.

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