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Template-directed dye-terminator incorporation (TDI) assay: a homogeneous DNA diagnostic method based on fluorescence resonance energy transfer

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ABSTRACT

A new method for DNA diagnostics based on template-directed primer extension and detection by fluorescence resonance energy transfer is described. In this method, amplified genomic DNA fragments containing polymorphic sites are incubated with a 5′-fluorescein-labeled primer (designed to hybridize to the DNA template adjacent to the polymorphic site) in the presence of allelic dye-labeled dideoxyribonucleoside triphosphates and a modified Taq DNA polymerase (Klentaq1-FY). The dye-labeled primer is extended one base by the dye-terminator specific for the allele present on the template. At the end of the genotyping reaction, the fluorescence intensities of the two dyes in the reaction mixture are analyzed directly without separation or purification. This homogeneous DNA diagnostic method, which we call the template-directed dye-terminator incorporation assay, is shown to be highly sensitive and specific and is suitable for automated genotyping of large numbers of samples.

INTRODUCTION

DNA analysis is becoming increasingly important in the diagnosis of hereditary diseases, detection of infectious agents, tissue typing for histocompatability, identification of individuals in forensic and paternity testing and monitoring the genetic make up of plants and animals in agricultural breeding programs (1). In addition, DNA analysis is crucial in large-scale genetic studies to identify susceptibility alleles associated with common diseases such as cardiovascular diseases (2), autoimmune disorders (3) and cancer (4). Since each of these applications involves the analysis of a large number of samples, simple, reliable and highly automated methods of DNA analysis are needed. Although simple tandem repeat polymorphisms (5) have been used successfully in molecular genetic studies, attention is now turning to single nucleotide polymorphisms (SNPs), the most common DNA sequence variation found in mammalian genomes (6). While most SNPs do not give rise to detectable phenotypes, a significant fraction of them are disease-causing mutations responsible for genetic diseases, including familial breast cancer (7) and hemochromatosis (8). As the DNA sequence of the human genome is completely elucidated, large-scale DNA analysis will play a crucial role in determining the relationship between genotype (DNA sequence) and phenotype (disease and health) (9). The prospect of large-scale DNA analysis using SNPs is hampered by the cumbersome, gel-based genotyping methods currently employed in their analysis. Even the relatively high throughput techniques, such as template-directed primer extension (10,11) and ligation assays (12,13), require immobilization of DNA followed by detection using either radioactive reporters or the multi-step enzyme linked immunosorbent assay (ELISA). The use of high density chip arrays for allele-specific hybridization analysis (14,15) and the homogeneous 5′-nuclease allele-specific oligonucleotide hybridization assay (TaqMan ASO; 16), both with considerable promise for high throughput, are still in the early stages of development.

We have developed a novel detection strategy that allows the rapid analysis of SNPs in a homogeneous assay, eliminating the need for product separation, use of radioactivity, the multi-step ELISA or specially designed and fabricated oligonucleotide chips. Our approach combines the specificity of enzymatic discrimination between the two alleles of an SNP in a template-directed primer extension reaction and the sensitivity of fluorescence resonance energy transfer.

Template-directed primer extension is a dideoxy chain terminating DNA sequencing protocol designed to ascertain the nature of the one base immediately 3′ of the sequencing primer, which is annealed to the target DNA immediately 5′ of the polymorphic site. In the presence of DNA polymerase and the appropriate dideoxyribonucleoside triphosphate (ddNTP), the primer is extended specifically by one base as dictated by the target DNA sequence at the polymorphic site. By determining which ddNTP is incorporated, the allele(s) present in the target DNA can be inferred. This genotyping method has been widely used and proven to be highly sensitive and specific (10,11).

Fluorescence resonance energy transfer (FRET) is observed when two fluorescent dyes are in close proximity and one fluorophore's emission spectrum overlaps the other's excitation spectrum (17). Energy transfer is mediated by dipole–dipole interaction, where the excitation energy absorbed by one dye

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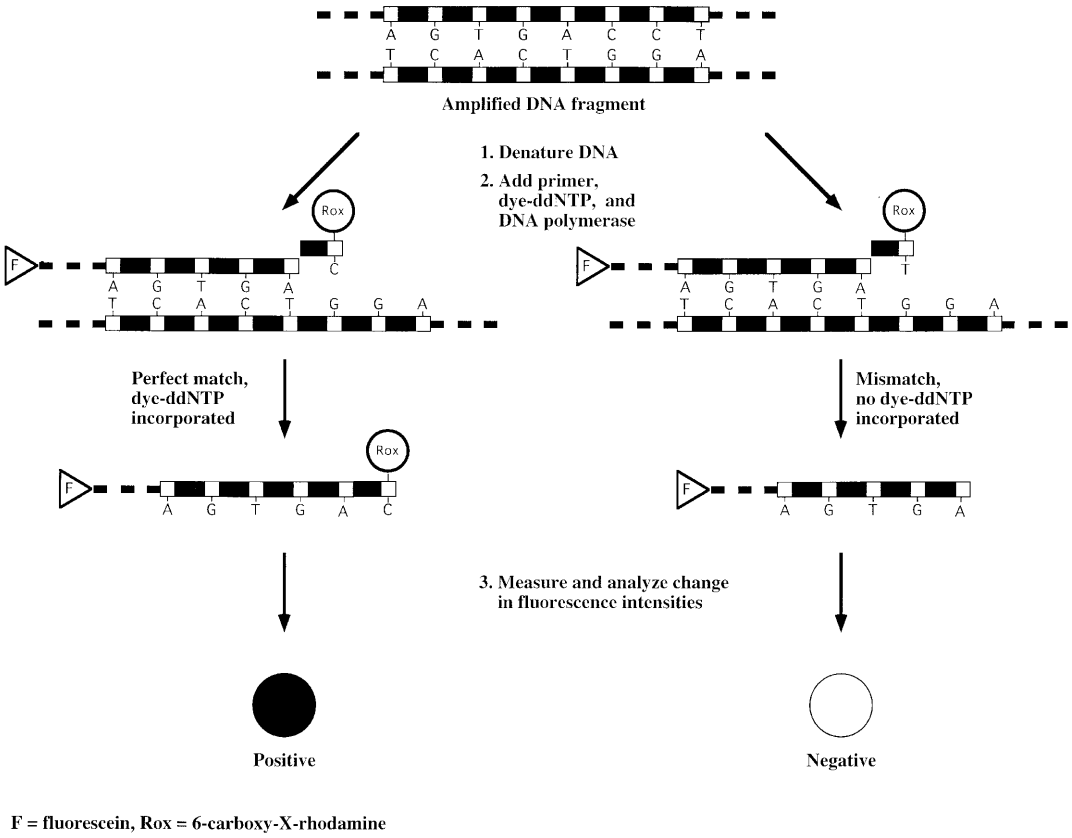

Figure 1. Schematic representation of the TDI genotyping assay. Amplified DNA fragments containing polymorphic sites are incubated with a fluorescein-labeled primer hybridizing to the DNA adjacent to the polymorphic site, the allelic ROX-labeled dideoxy terminator and the Klentaq1-FY DNA polymerase. At the end of the genotyping reaction, the reaction mixture is analyzed for changes in fluorescence intensities.

(donor), instead of being emmitted as fluorescence, is transferred to the second dye (acceptor) by resonance. Spectroscopically, when the donor is excited, its specific emission intensity decreases (quenched), while the specific emission intensity of the acceptor 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. The efficiency of FRET is very sensitive to the distance between the two fluorophores. When the distance between the two dyes increases the efficiency of energy transfer decreases dramatically. In fact, FRET efficiency is inversely proportional to the 6th power of the distance between the donor and acceptor dyes (17). For fluorophores placed on an oligonucleotide in free solution, however, the spatial distance between the two dyes is more important than the linear distance separating them on the oligonucleotide. This is the reason why FRET has been observed when two dyes are placed 20 bases apart on an oligonucleotide (18). The ability to detect intramolecular FRET against a background of intermolecular FRET provides a novel and unique detection system that requires no separation or purification of the product in DNA diagnostic assays such as primer extension reactions. FRET can be measured either by quenching of the donor emission, the increase in acceptor emission, or both. FRET has been exploited successfully in automated DNA sequencing using energy transfer dye primers (19), in 5′-nuclease allele-specific oligonucleotide hybridization assays (TaqMan ASO; 18), in detecting DNA hybridization (20) and in the study of protein–protein interaction (21).

In our method, named the template-directed dye-terminator incorporation (TDI) assay, the sequencing primer is 5′-labeled with the donor dye (fluorescein) and the ddNTPs are labeled with an acceptor dye (6-carboxy-X-rhodamine, ROX). FRET occurs when the dye-labeled ddNTP is incorporated onto the sequencing primer in the presence of DNA polymerase and target DNA. The genotype of the target DNA molecule can be determined simply by exciting the fluorescein dye on the sequencing primer and seeing if the acceptor dye exhibits FRET (Fig. 1).

In this report, we demonstrate the utility of FRET as a simple, sensitive and specific detection method in a homogeneous primer extension genotyping assay. Both single-stranded synthetic DNA oligomers and double-stranded DNA fragments amplified by PCR (22) can be used as templates in this assay. In all cases, FRET proves to be highly sensitive and specific in the TDI assay.

MATERIALS AND METHODS

Enzymes

AmpliTaq DNA polymerase was purchased from Perkin Elmer Corporation (Foster City, CA) and Klentaq1-FY DNA polymerase was purchased from the laboratories of Dr Wayne Barnes (Washington University, St Louis, MO).

Table 1. Oligonucleotide sequences

Oligomer	Sequence
s14102-40A	5'-ATTTTACAAAAATAAAACAAAGAAACCACTAAGCCATAAA
s14102-40C	5'-ATTTTACAAAAATAAAACAACGAAACCACTAAGCCATAAA
s14102-40G	5'-ATTTTACAAAAATAAAACAAGGAAACCACTAAGCCATAAA
s14102-40T	5'-ATTTTACAAAAATAAAACAATGAAACCACTAAGCCATAAA
s14102-F	5'-F-TTTATGGCTTAGTGGTTTC
D18S8-p1	5'-TTGCACCATGCTGAAGATTGT
D18S8-p2	5'-ACCCTCCCCCTGATGACTTA
D18S8-F	5'-F-CACTGCAAGCTCTGCCTCC
DXS17-p1	5'-GGTACATGACAATCTCCCAATAT
DXS17-p2	5'-GCAATTATCTGTATTACTTGAAT
DXS17-F	5'-F-CCCTTATGCACTTATCCTT

Oligonucleotides

Oligonucleotides used are listed in Table 1. PCR and TDI primers were obtained commercially (GENSET Corporation, La Jolla, CA). The TDI primers were 5′-labeled with fluorescein and purified by reverse phase high performance liquid chromatography by the supplier. Synthetic templates s14102-40A, s14102-40C, s14102-40G and s14102-40T were synthesized by the Genome Sequencing Center at Washington University (St Louis, MO).

PCR amplification

Human genomic DNA (20 ng) from 40 unrelated individuals was amplified in 40 µl reaction mixtures 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 each primer and 2 U AmpliTaq DNA polymerase. The reaction mixture was held at 94°C for 3 min, followed by 10 cycles of 94°C for 10 s, ramping to 60°C over 90 s, held at 60°C for 30 s, followed by 30 cycles of 94°C for 10 s and 53°C for 30 s. At the end of the reaction, the reaction mixture was cooled to 4°C to await further manipulations.

Gel purification of PCR products

The PCR products resulting from amplifying the sequence-tagged sites D18S8 (367 bp) and DXS17 (620 bp) were gel purified by running the samples on a 1% low melting point agarose gel in 1× TAE. After staining with ethidium bromide, the gel bands containing the PCR products were excised under long wavelength (365 nm) UV transillumination. The DNA was extracted from the gel slice using the Wizard PCR purification system (Promega, Madison, WI) according to the manufacturer's instructions.

Dideoxyribonucleoside triphosphates

Dideoxyribonucleoside triphosphates labeled with ROX (ROX–ddA, ROX–ddC, ROX–ddG and ROX–ddU) were obtained from DuPont NEN (Boston, MA). Unlabeled ddNTPs were purchased from Pharmacia Biotech (Piscataway, NJ).

Genotyping by the TDI assay

Genotyping reactions were performed in 20 µl mixtures containing 50 mM Tris–HCl, pH 9.0, 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, 8% glycerol, 0.1% Triton X-100, 25 nM dye-labeled TDI primer, 100 nM ROX–ddNTP and DNA template (>50 nM synthetic 40mer or PCR products). (Note: 250 nM unlabeled ddNTPs were also added to the reaction mixture in the reactions using synthetic oligonucleotide templates.) The reaction mixtures were incubated at 93°C for 1 min, followed by 35 cycles of 93°C for 10 s and 50°C for 30 s. The reaction was stopped by the addition of 10 µl 50 mM EDTA, pH 9.0.

Analysis of TDI reaction products by GeneScan gel electrophoresis

After the TDI reaction, 1.0 µl reaction mixture was added to 5 µl loading buffer (98% formamide, 10 mM EDTA). An aliquot of this mixture (2.0 µl) was loaded onto a sequencing gel (6% polyacrylamide, 8 M urea, 1× TBE) for electrophoresis on an Applied Biosystems 373A automatic DNA sequencer (PE-ABD). The fluorescent species were analyzed using the GeneScan 672 software (PE-ABD).

Analysis of TDI reaction products by fluorescence spectroscopy

The TDI reaction mixture was denatured and diluted by the addition of 125 µl 0.2 N NaOH. The diluted reaction mixtures were transferred to a 96-well white microplate (PE-ABD) and the fluorescence emission determined with a Luminescence Spectrometer LS-50B (PE-ABD). The excitation wavelength was set at 488 nm to determine the emission intensity of fluorescein (FF, 515 nm detection wavelength) and that of ROX with enhanced emission due to energy transfer (FR, 605 nm). The excitation wavelength was set at 580 nm to determine the intrinsic emission intensity of ROX (RR, 605 nm). Fluorescence enhancement (FE) is defined as FR/(RR × FF). A sample is scored as positive for a given allele when it's fluorescence enhancement value (FE_S) is significantly higher than that of the controls (FE_C) at the >99% confidence level. Depending on the particular ROX–ddNTP used and the particular experiment performed, significance at this level of confidence is achieved when FE_S is greater than FE_C by 15–25%.

RESULTS AND DISCUSSION

While 5′-dye-labeled primers and dye-labeled dideoxy-terminators have been used extensively in sequencing reactions (23,24) and the sensitivity and specificity of template-directed primer extension genotyping methods are well established when single-stranded templates are used (10,11), the use of FRET as a detection method in a primer extension reaction using double-stranded templates has not been reported prior to this work. Furthermore, we used a member of a new class of mutant Taq DNA polymerase (25) that incorporates dye-labeled ddNTPs much more efficiently than the wild-type Taq DNA polymerase in our assay, instead of the Klenow fragment of *Escherichia coli* DNA polymerase I or T7 DNA polymerase commonly used in primer extension reactions (10,11). Two sets of experiments were performed to show that FRET is a simple, highly sensitive and specific detection method in a primer extension reaction for single base pair changes. In the first set of experiments, four synthetic templates containing the four possible nucleotides at one particular site in the middle of otherwise identical oligonucleotides were used to establish the sensitivity and specificity of FRET detection of dye-terminator incorporation. In the second set of experiments, 80 PCR products amplified from genomic DNA were used as templates in the TDI assay to show that accurate genotyping data were produced efficiently by this assay.

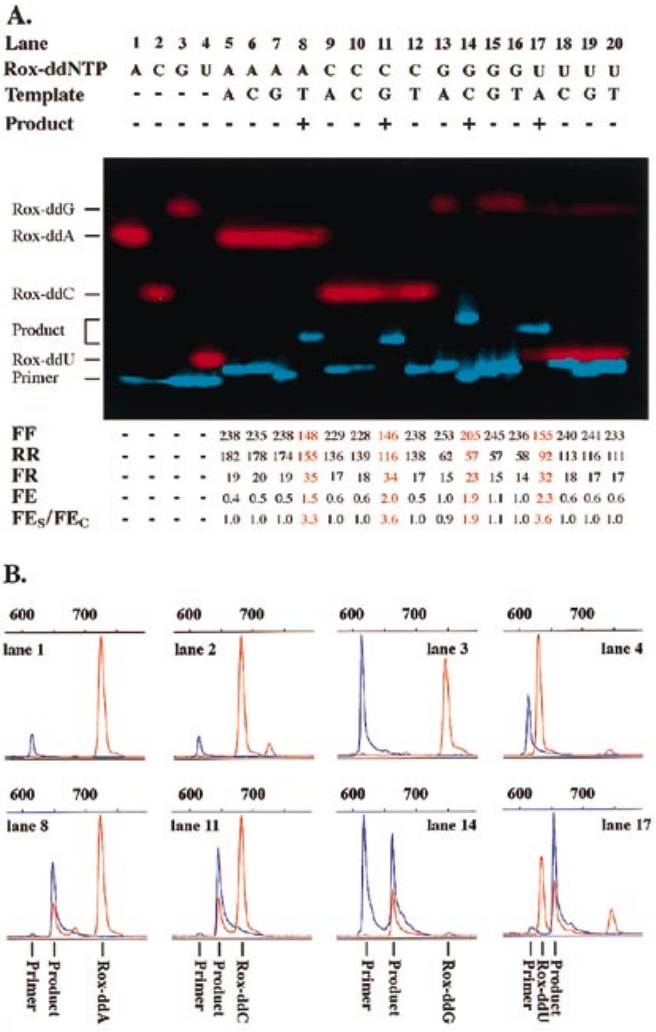

Figure 2. Genotyping of synthetic DNA templates by the TDI assay. (**A**) GeneScan image of the genotyping assay together with the fluorescence intensity reading of the various reaction mixtures. Blue signals in lanes 1–4 represent the fluorescein-labeled primer. Red signals in all lanes represent unreacted ROX-labeled terminators. The faint red bands found in lanes 17–20 are due to degraded ROX–ddU; similar faint red signals are found high in the other reaction lanes but are outside the image window presented in this figure. The blue bands in positive lanes 8, 11, 14 and 17 represent doubly labeled products in which the strong fluorescein signals mask the red ROX signal underneath. The blue signals in the remaining lanes (5–7, 9–10, 12–13, 15–16 and 18–20) are singly labeled products with specific incorporation of unlabeled ddNTPs. Fluorescence readings of all the reaction mixtures are listed under the image. FF, emission at 515 nm upon excitation at 488 nm; RR, emission at 605 nm upon excitation at 580 nm; FR, emission at 605 nm upon excitation at 488 nm; FE = [FR]/[RR × FF]; FE_S, the value of FE for the test sample; FE_C, the value of FE for the control sample. The fluorescence intensities of the positive reaction mixtures are in red and, except for the $[RR]_{ddG}$ values, are significantly different from their counterparts in the negative reaction mixtures. (**B**) Chromatograms of the GeneScan image comparing the control mixtures (lanes 1–4) with the corresponding positive reactions (lanes 8, 11, 14 and 17). In each instance, the product peak shows coincident fluorescence due to both fluorescein and ROX that are found on the same molecule.

DNA typing by the TDI assay using synthetic templates

A set of synthetic 40mers with identical sequence except for position 21, where each of the four possible bases were represented in each of the four different synthetic templates (shown in bold in Table 1), was prepared. Each synthetic 40mer served as template in four separate reactions where it was incubated with the 5′-fluorescein-labeled sequencing primer and one of the four dye-labeled terminators in the presence of Klentaq1-FY and the other three unlabeled terminators. At the end of the TDI reaction, an aliquot of the mixture was analyzed directly by electrophoresis on a sequencing gel using the GeneScan software and the remainder of the mixture was analyzed directly by fluorescence spectrophotometry. As shown in Figure 2A, the GeneScan gel image confirmed that only the one base that was perfectly complementary to the specific template was incorporated in each reaction. This is clearly seen because fluorescein-labeled oligomers migrate slightly differently depending on the terminator incorporated. For example, the fluorescein signals for lanes 12, 16 and 20 (where unlabeled ddA was incorporated) migrated the same distance in the gel. The same was observed for the signals in lanes 7, 15 and 19 (ddC incorporation), lanes 6, 10 and 18 (ddG incorporation) and lanes 5, 9 and 13 (ddT incorporation). When a dye-terminator was incorporated, the fluorescein and ROX signals co-migrated and were retarded due to the added (less polar) dye moiety (lanes 8, 11, 14 and 17). Figure 2B shows the chromatograms of control lanes 1–4 and the positive reaction lanes 8, 11, 14 and 17, where the co-migrated fluorescein and ROX signals are clearly seen. It is interesting to note that only ~50% of primers had ROX–ddG incorporation (Fig. 2A, lane 14) due to complete consumption of ROX–ddG.

Fluorescence spectrophotometric analysis of the reaction mixture at the end of the TDI assay showed that three types of changes in fluorescence intensity were observed when dye-terminators were incorporated. The first two types of changes were seen when the reaction mixture was excited by light at the fluorescein-specific absorption wavelength (488 nm), namely a reduction in fluorescein-specific emission (FF) due to quenching by the incorporated dye and an increase in ROX-specific emission (FR) due to FRET. The third type of change was observed when the mixture was excited by light at the ROX-specific absorption wavelength (580 nm): a reduction in acceptor ROX

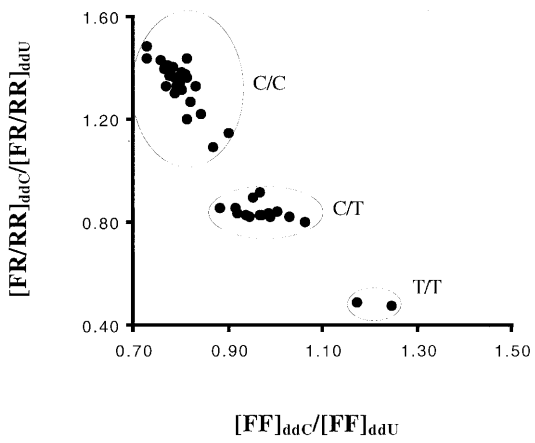

Figure 3. Scatter plot of the TDI fluorescence data for 40 unrelated individuals with respect to the polymorphic marker D18S8. The $[FR/RR]_{ddC}$:$[FR/RR]_{ddU}$ ratio is plotted against the $[FF]_{ddC}$:$[FF]_{ddU}$ ratio. The data points fall into three distinct categories: (i) high $[FR/RR]_{ddC}$:$FR/RR]_{ddU}$ ratio but low $[FF]_{ddC}$:$[FF]_{ddU}$ ratio, representing samples with the homozygous C/C genotype; (ii) low $FR/RR]_{ddC}$:$[FR/RR]_{ddU}$ ratio but high $[FF]_{ddC}$:$[FF]_{ddU}$ ratio, representing samples with the homozygous T/T genotype; (iii) intermediate values for the two ratios, representing samples with the heterozygous C/T genotype.

Table 2. TDI assay data for diallelic marker DXS17[a]

	ROX-ddC				ROX-ddU				
Sample	FF	FR/RR	FE	FE_S/FE_C	FF	FR/RR	FE	FE_S/FE_C	Genotype
1	239	0.17	0.07	0.84	178	0.35	0.20	1.84	T/T
2	160	0.32	0.20	2.29	172	0.31	0.18	1.68	C/T
3	143	0.37	0.26	3.02	203	0.20	0.10	0.90	C/C
4	208	0.19	0.09	1.05	136	0.44	0.32	3.00	T/T
5	174	0.28	0.16	1.84	203	0.19	0.10	0.89	C/C
6	220	0.18	0.08	0.95	134	0.47	0.35	3.26	T/T
7	214	0.18	0.08	0.98	133	0.43	0.33	3.05	T/T
8	150	0.39	0.26	3.00	149	0.40	0.27	2.52	C/T
9	132	0.42	0.32	3.66	199	0.20	0.10	0.93	C/C
10	214	0.18	0.08	0.98	138	0.42	0.31	2.89	T/T
11	214	0.18	0.08	0.97	127	0.48	0.38	3.54	T/T
12	213	0.18	0.09	0.99	132	0.44	0.33	3.11	T/T
13	208	0.19	0.09	1.04	145	0.36	0.25	2.33	T/T
14	154	0.35	0.23	2.63	157	0.36	0.23	2.17	C/T
15	214	0.18	0.09	0.99	200	0.19	0.10	0.91	-
16	126	0.44	0.35	4.00	200	0.20	0.10	0.95	C/C
17	207	0.17	0.08	0.95	143	0.40	0.28	2.61	T/T
18	216	0.17	0.08	0.92	136	0.44	0.32	3.03	T/T
19	207	0.17	0.08	0.95	176	0.24	0.14	1.28	T/T
20	139	0.38	0.27	3.15	197	0.20	0.10	0.93	C/C
21	174	0.25	0.14	1.63	189	0.19	0.10	0.94	C/C
22	190	0.25	0.13	1.51	200	0.19	0.10	0.91	C/C
23	167	0.30	0.18	2.10	193	0.19	0.10	0.94	C/C
24	175	0.28	0.16	1.83	196	0.20	0.10	0.95	C/C
25	183	0.25	0.14	1.61	203	0.20	0.10	0.91	C/C
26	199	0.19	0.10	1.10	195	0.21	0.11	0.99	-
27	189	0.22	0.12	1.37	201	0.19	0.10	0.90	C/C
28	172	0.27	0.16	1.80	194	0.20	0.10	0.96	C/C
29	207	0.18	0.08	0.98	160	0.32	0.20	1.87	T/T
30	195	0.23	0.12	1.34	182	0.25	0.14	1.29	C/T
31	193	0.20	0.10	1.19	138	0.39	0.28	2.62	T/T
32	159	0.35	0.22	2.51	205	0.20	0.10	0.92	C/C
33	209	0.18	0.09	0.99	155	0.32	0.21	1.94	T/T
34	213	0.17	0.08	0.94	142	0.39	0.27	2.54	T/T
35	139	0.38	0.27	3.17	167	0.21	0.13	1.19	C/C
36	212	0.18	0.08	0.96	136	0.41	0.30	2.83	T/T
37	190	0.19	0.10	1.16	158	0.23	0.14	1.35	T/T
38	165	0.33	0.20	2.31	158	0.34	0.22	2.04	C/T
39	209	0.18	0.08	0.98	146	0.39	0.27	2.50	T/T
40	155	0.33	0.21	2.43	151	0.35	0.23	2.18	C/T
control 1	213	0.18	0.09		193	0.21	0.11		
control 2	214	0.18	0.08		196	0.20	0.10		
control 3	205	0.17	0.08		192	0.20	0.10		
control 4	211	0.18	0.08		193	0.20	0.11		
control 5	200	0.18	0.09		189	0.20	0.11		
control 6	204	0.18	0.09		190	0.21	0.11		
contorl 7	201	0.18	0.09		188	0.19	0.10		
control 8	205	0.18	0.09		188	0.21	0.11		

[a]See Figure 2 legend for definition of abbreviations.

(RR) emission due to quenching by the DNA oligomer to which the acceptor had attached. The fluorescence readings for each lane in Figure 2A show these changes clearly and in each of the three intensity changes, the difference between the positive and negative reactions was highly significant, with the exception of the RR reading for ROX–ddG. The FE_S:FE_C ratios for the positive assays ranged from 190 to 360%.

Based on a series of dilution experiments we performed using the single-stranded synthetic templates, highly significant differences were found between positive and negative reactions at >5 nM template concentrations (data not shown).

DNA typing by the TDI assay using PCR products as templates

To demonstrate that double-stranded DNA samples could be used as templates in the TDI assay, genomic DNA derived from 40 unrelated individuals was amplified and the genotype determined by the TDI assay. Two polymorphic sequence-tagged sites, DXS17 (26) and D18S8 (27), were used to validate our assay. Gel purified PCR products amplified from genomic DNA of 40 unrelated individuals served as templates and were placed in two parallel TDI reactions containing the fluorescein-labeled sequencing primer and one of the allelic ROX-labeled terminators. As shown in Table 2, all but two samples among the 40 tested for the DXS17 locus gave definitive genotypes with the positive threshold set at $FE_S:FE_C > 1.25$. The two DNA samples that yielded no definitive genotypes were analyzed by agarose gel electrophoresis and were shown to have very weak product bands, indicating suboptimal PCR amplification to be the reason for the false negative results. For the D18S8 locus, all 40 DNA samples were amplified successfully and the PCR products were analyzed in the TDI assay as before. Table 3 shows that all 40 samples yielded definitive genotypes when the positive threshold is set at $FE_S:FE_C > 1.15$. These results can be visualized by plotting the ratio of FF between the two alleles against the ratio FR:RR between the two alleles (Fig. 3). In the reaction containing the dye-terminator specific for allele 1, a sample homozygous for allele 1 displays reduced FF and RR values due to quenching and an enhanced FR value due to FRET. In contrast, in the reaction containing the dye-terminator specific for allele 2, a sample homozygous for allele 1 displays minimal change in all three values as compared with negative controls. A sample homozygous for allele 1 (ddC) therefore has a high $(FR:RR)_1/(FR:RR)_2$ value and a low $(FF)_1/(FF)_2$ value (left upper corner of the plot in Fig. 3). Similar

Table 3. TDI assay data for diallelic marker D18S8[a]

	ROX-ddC				ROX-ddU				
Sample	FF	FR/RR	FE	FE_S/FE_C	F	FR/RR	FE	FE_S/FE_C	Genotype
1	231	0.30	0.13	1.35	238	0.36	0.15	1.19	C/T
2	185	0.37	0.20	2.09	235	0.28	0.12	0.96	C/C
3	191	0.38	0.20	2.09	238	0.28	0.12	0.92	C/C
4	207	0.33	0.16	1.66	230	0.29	0.12	0.99	C/C
5	188	0.40	0.21	2.24	231	0.28	0.12	0.96	C/C
6	197	0.37	0.19	1.96	250	0.27	0.11	0.85	C/C
7	187	0.38	0.20	2.13	236	0.29	0.12	0.96	C/C
8	218	0.32	0.15	1.53	217	0.38	0.17	1.38	C/T
9	192	0.38	0.20	2.10	245	0.27	0.11	0.88	C/C
10	235	0.22	0.09	1.00	200	0.46	0.23	1.82	T/T
11	190	0.35	0.19	1.95	231	0.28	0.12	0.96	C/C
12	192	0.38	0.20	2.05	237	0.27	0.12	0.91	C/C
13	176	0.41	0.23	2.44	232	0.29	0.12	0.97	C/C
14	188	0.37	0.20	2.06	243	0.28	0.11	0.91	C/C
15	186	0.37	0.20	2.11	239	0.27	0.11	0.90	C/C
16	202	0.33	0.16	1.70	212	0.37	0.17	1.36	C/T
17	181	0.39	0.21	2.24	234	0.27	0.12	0.93	C/C
18	176	0.39	0.22	2.33	241	0.27	0.11	0.89	C/C
19	193	0.33	0.17	1.76	205	0.40	0.19	1.54	C/T
20	169	0.43	0.25	2.65	231	0.29	0.12	0.98	C/C
21	187	0.35	0.19	1.95	204	0.42	0.21	1.63	C/T
22	186	0.31	0.17	1.76	211	0.36	0.17	1.37	C/T
23	194	0.32	0.16	1.72	212	0.37	0.18	1.39	C/T
24	195	0.33	0.17	1.75	208	0.39	0.19	1.50	C/T
25	221	0.31	0.14	1.48	228	0.34	0.15	1.19	C/T
26	212	0.30	0.14	1.48	214	0.36	0.17	1.35	C/T
27	192	0.37	0.19	2.02	236	0.27	0.12	0.91	C/C
28	198	0.31	0.16	1.65	204	0.38	0.19	1.47	C/T
29	182	0.41	0.23	2.37	237	0.29	0.12	0.98	C/C
30	188	0.39	0.21	2.16	236	0.29	0.12	0.97	C/C
31	205	0.31	0.15	1.57	236	0.28	0.12	0.94	C/C
32	204	0.32	0.16	1.64	207	0.38	0.18	1.47	C/T
33	211	0.30	0.14	1.51	205	0.37	0.18	1.43	C/T
34	191	0.36	0.19	1.98	226	0.30	0.13	1.04	C/C
35	174	0.39	0.22	2.33	224	0.28	0.12	0.98	C/C
36	237	0.22	0.09	0.97	190	0.47	0.24	1.94	T/T
37	181	0.39	0.21	2.24	226	0.29	0.13	1.03	C/C
38	189	0.37	0.20	2.06	227	0.28	0.12	0.98	C/C
39	212	0.30	0.14	1.50	200	0.38	0.19	1.50	C/T
40	184	0.34	0.19	1.96	227	0.29	0.13	1.01	C/C
control 1	235	0.22	0.09		234	0.29	0.12		-
control 2	244	0.22	0.09		243	0.29	0.12		-
control 3	234	0.22	0.09		226	0.30	0.13		-
control 4	230	0.23	0.10		230	0.29	0.13		-
control 5	232	0.23	0.10		229	0.28	0.12		-
control 6	231	0.22	0.10		229	0.29	0.13		-
control 7	229	0.22	0.10		227	0.29	0.13		-
control 8	228	0.22	0.10		222	0.29	0.13		-

[a]See Figure 2 legend for definition of abbreviations.

arguments predict that a sample homozygous for allele 2 (ddU) will have a low $(FR:RR)_1/(FR:RR)_2$ value and a high $(FF)_1/(FF)_2$ value (right lower corner of the plot in Fig. 3). Heterozygous samples have intermediate values (close to 1.0) for both ratios and occupy the region in between the two extremes in the plot. The genotypes for all 78 DNA samples determined by the TDI assay are in perfect concordance with those determined by the highly accurate oligonucleotide ligation assay (12).

Based on these and other results, the false negative rate of the TDI assay is estimated to be ~5%, with all failures attributable to suboptimal PCR amplification leading to DNA concentrations below the detection limit. The false positive rate is ~1.5%, with contamination being the cause for such results (data not shown).

We have previously shown that Taq DNA polymerases incorporate dye-terminators at a variable rate, depending on the local sequence context, in the presence of dNTPs (28,29). Because there are no dNTPs in the TDI assay to compete with the dye-terminators, local sequence context imposes no noticeable influence on the assay. This is the case for the marker DXS17, where the two alleles are T and C following the base string TT. Based on our previous work on dye-terminator sequencing peak height patterns using the Klentaq1-FY family of DNA polymerase, T incorporation following TT is inefficient while C incorporation following TT is highly efficient in the presence of dNTPs, giving C peaks that are two to three times the size of T peaks in the sequencing chromatogram (29). For the six DNA samples heterozygous for DXS17 (samples 2, 8, 14, 30, 38 and 40, Table 3), however, the average FE_S:FE_C values for ROX–ddC and ROX–ddU are 2.33 and 1.98 respectively, a 15% difference that is attributable to the difference in fluorescence properties of the dye-terminators rather than to a difference in the efficiency of dye-terminator incorporation.

Demands for genetic testing (i.e. assaying for the presence or absence of known DNA polymorphisms or mutations) are expected to increase dramatically in the areas of diagnostics, forensics and population studies. For example, population studies involving thousands of individuals and hundreds of markers were performed recently to localize genes important in the development of type I diabetes mellitus (30). A homogeneous genotyping assay is highly suitable for large-scale genetic studies because it is not limited by a particular reaction format and it offers the flexibility of using the best markers as they become available for a particular application without redesigning or refabricating high density DNA chips. Furthermore, the TDI assay is simple to set up (by adding the standard reagent mixture to the DNA template), the results are obtained in electronic form minutes after the

allele-discriminating reaction is performed and the genotype can be assigned automatically by the use of a simple computer program.

With proof of the principle of FRET detection completed, studies are now underway to combine PCR and TDI to achieve a one-tube reaction without the need for purification of PCR products from the unreacted primers and dNTPs, which will interfere with the TDI reaction. Moreover, a number of other dyes (including TAMRA, JOE and Texas Red) can act as acceptors for fluorescein, making it possible to perform TDI for both alleles in the same reaction vessel. Furthermore, since the principle of FRET applies to any donor–acceptor pair, including those absorbing in the infrared region, one can utilize near infrared donor–acceptor pairs that can be assayed in commonly used plastic ware with no concern for interfering background emissions. As DNA diagnostic tests will no doubt be performed more and more by clinical rather than research laboratories, methods (such as the TDI assay) utilizing standard protocols that require minimal laboratory skills or manual handling will be crucial to the clinical practice of medicine in the future.

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