Technical Advance

Hybridization-Induced Dequenching of Fluorescein-Labeled Oligonucleotides

A Novel Strategy for PCR Detection and Genotyping

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Fluorescence-based detection methods are being increasingly utilized in molecular analyses. Sequence-specific fluorescently-labeled probes are favored because they provide specific product identification. The most established fluorescence-based detection systems employ a resonance energy transfer mechanism effected through the interaction of two or more fluorophores or functional groups conjugated to oligonucleotide probes. The design, synthesis and purification of such multiple fluorophore-labeled probes can be technically challenging and expensive. By comparison, single fluorophore-labeled probes are easier to design and synthesize, and are straightforward to implement in molecular assays. We describe herein a novel fluorescent strategy for specific nucleic acid detection and genotyping. The format utilizes an internally quenched fluorescein-oligonucleotide conjugate that is subsequently dequenched following hybridization to the target with an attendant increase in fluorescence. Reversibility of the process with strand dissociation permits T_m-based assessment of bp complementarity and mismatches. Using this approach, we demonstrated specific detection, and discrimination of base substitutions of a variety of synthetic nucleic acid targets including Factor V Leiden and methylenetetrahydrofolate reductase. We further demonstrated compatibility of the novel chemistry with polymerase chain reaction by amplification and genotyping of the above listed loci and the human hemoglobin β chain locus. In total, we analyzed 172 clinical samples, comprising wild-type, heterozygous and homozygous mutants of all three loci, with 100% accuracy as confirmed by DNA sequencing, established dual hybridization probe or high performance liquid chromatography-based methods. Our results indicate that the

dequenching-based single fluorophore format is a feasible strategy for the specific detection of nucleic acids in solution, and that assays using this strategy can provide accurate genotyping results. (Am J Pathol 2003, 163:29–35)

Polymerase chain reaction (PCR)-based detection of nucleic acids is increasingly being used in molecular diagnostics and research. Traditionally, the experimental protocols have entailed a discontinuous two-step process involving amplification of target sequences and subsequent product detection by ultraviolet transillumination of ethidium-bromide-stained gels, chemiluminescent, or radioisotopic detection. The advent of homogeneous assays in which both target amplification and detection are performed simultaneously in a closed-tube setting, has several advantages favoring its utilization in molecular assays. These include the minimization of the risk of contamination inherent in the closed-tube format, and the faster turnaround time due to the lack of a postamplification analytical step. Fluorescence-based schemes are the favored method for nucleic acid detection in such assays.

The fluorescence chemistries used in nucleic acid detection comprise those incorporating non-specific double-stranded DNA (dsDNA) binding dyes, or those using fluorescently-labeled oligonucleotide probes that hybridize specifically to sequences within the target. The non-specific dsDNA binding dyes include ethidium bromide,¹ YO-PRO 1,² and SYBR Green I.³ The binding of dsDNA dyes to double-stranded DNA is accompanied by a dramatic increase in fluorescence, thus the non-specific

Supported by grant CA83984 from the National Institutes of Health to K.S.J.E.-J., and by the ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah.

Accepted for publication March 19, 2003.

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Table 1. Oligonucleotides Utilized for Fluorescence Melting Curve Analyses for Artificial Templates

Oligonucleotide	Sequence	GenBank accession no.	Base pair position
Templates			
Factor V wild-type	5'-AATACCTGTATTCTCGCCTGTCCAGGG-3'	L32764	281–254
Factor V mutant	5'-AATACCTGTATTCT T GCCTGTCCAGGG-3'	L32764	281–254
MTHFR wild-type	5'-GATGATGAAATCGGCTCCCGCAGACACCTTCT-3'	AF105980	142–111
MTHFR mutant	5'-GATGATGAAATCG A CTCCCGCAGACACCTTCT-3'	AF105980	142–111
Probes			
Factor V probe	5'-ACAGGCAAGGAATACAGG-Fluor.-3'	L32764	260–277
MTHFR probe	5'-Fluor.-GGTGTCTGCGGGAGTCGATT-P-3'	AF105980	115–134

Fluor, fluorescein; P, phosphate; Base substitutions bolded and underlined.

dsDNA binding dye-based methods are capable of detecting amplification and product accumulation, but are unable to provide unambiguous verification of the identity of the amplified product. The probe-based methods confer additional specificity to the detection of the amplification products^{3–5} and are capable of distinguishing products differing by only one base.^{6–8} By comparison, the sequence-specific fluorescent probe-based methods include those using adjacent hybridization probes,^{3,7} exonuclease (TaqMan),⁹ hairpin (Molecular beacon),⁴ and self-probing amplicons (Scorpion).¹⁰ In general, the sequence-specific methodologies entail a fluorescence quenching or potentiation interaction between two or more fluorophores,^{4,11,12} or other more complex interactions involving additional functional groups.¹³ The synthesis of such dual- or multiple-fluorophore or chemical-group-labeled oligonucleotides for real-time PCR can be technically demanding and expensive. Hence, it is desirable to develop less complex approaches for PCR product detection.

Sequence-specific probe-based designs using only one fluorophore are one method for simplification of fluorescence-based PCR product detection, and represent a significant technical advance over dual-fluorophore based systems. Here, we describe a novel strategy for fluorescence detection and genotyping of PCR products. The method exploits the phenomenon of dequenching of fluorescence of fluorescein-labeled oligonucleotides on hybridization to a complementary DNA target. The reversibility of the phenomenon enables the performance of melting curve analysis, which permits genotyping by *T_m*. Our novel method yielded 100% concordance when compared with standard mutation detection assays. To our knowledge, this is the first study describing the exploitation of the dequenching phenomenon for PCR detection and genotyping, and establishes the utility of this phenomenon for PCR detection in general.

Materials and Methods

DNA Samples

Artificial Templates

Oligonucleotides containing sequences from the Factor V gene or the methylenetetrahydrofolate reductase (MTHFR) gene were synthesized by Operon Technologies (Alameda, CA). The Factor V sequence included the Leiden mutation (G1691A) and the MTHFR sequence

included the C677T base substitution. The oligonucleotide sequences consisted of either the wild-type or the mutant sequence as listed in Table 1.

Clinical Samples

For genotypic analysis of the human Factor V, MTHFR and β -globin loci, DNA was extracted from leukocytes obtained from whole blood samples using the MagNa Pure LC Instrument (Roche Molecular Biochemicals, Indianapolis, IN). All samples were obtained from the archived inventories of ARUP Laboratories (Salt Lake City, UT) with institutional review board approval. For the Factor V locus, we tested 65 wild-types, 23 heterozygotes (G1691A), and 12 homozygotes (G1691A). For the MTHFR locus, we tested 10 wild-types, 5 heterozygotes (C677T), and 5 homozygotes (C677T). For the human β -globin locus, we tested DNA from 37 wild-types (HbAA), 5 HbSS (homozygous), 3 HbCC (homozygous), and 7 HbSC (compound heterozygous) samples by dequenching PCR. All of the genotypes were confirmed by DNA sequencing and/or a modification of a previously described protocol for Factor V Leiden,⁶ MTHFR,⁸ and hemoglobin β -chain genotyping by high performance liquid chromatography (HPLC).¹⁴ The dequenching-based genotyping studies were scored independently from the DNA sequencing, dual-fluorescent probe and HPLC studies, and the concordance between the results of the various approaches was determined after dequenching PCR.

Fluorescence Melting Curve Analysis

Oligonucleotide probes labeled with fluorescein at either the 5' or 3' end were synthesized by Operon Technologies. Probe sequences for the Factor V and MTHFR genes are listed in Table 1. The fluorescein label was attached to the oligonucleotide probe with an intervening six-carbon spacer. Probes were designed to be complementary to the mutant sequences because this configuration resulted in the greatest *T_m* shifts from mismatches for both the Factor V and MTHFR genotyping assays.

Melting curve analysis was performed using the Light-Cycler (Roche Molecular Biochemicals). Each 10 μ L reaction contained 0.4 μ M/L template oligonucleotide, 0.2 μ M/L fluorescein-labeled probe, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 3.0 mmol/L MgCl₂, and 250 mg/ml

Table 2. Primers and Probes Used for PCR Amplification and Genotyping of Clinical Samples

Oligonucleotide	Sequence	GenBank accession no. or literature reference	Base pair position
Factor V			
Forward primer	5'-GAGAGACATCGCCTCTGGGCTA-3'	L32764	201-222
Reverse primer	5'-TGTTATCACACTGGTGCTAA-3'	PR-990 (reference no. 16)	127-146 (intron 10)
Dequenching probe	5'-ACAGGCAAGGAATACAGG-Fluor.-3'	L32764	260-277
MTHFR			
Forward primer	5'-TGGCAGGTTACCCCAAA-3'	AF105980	45-61
Reverse primer	5'-CATGTCGGTGCATGCCTTCA-3'	AF105980	202-183
Dequenching probe	5'-Fluor.-GGTGTCTGCGGGAGTCGATT-P-3'	AF105980	115-134
β-Globin			
Forward primer	5'-ACACAACGTGTTCCTAGC-3'	AF105973	117-136
Reverse primer	5'-CAACTTCATCCACGTTCCACC-3'	AF105973	226-207
Dequenching probe	5'-GACTTCTCCACAGGAGTCAGG-Fluor.-3'	AF105973	182-162

Fluor, fluorescein; P, phosphate.

Figure 1. A: Fluorescence (F) versus temperature (T) curves for sequence-specific dequenching of fluorescein-labeled oligonucleotides complementary to the Factor V gene sequence. Melting protocols entailed initial denaturation to 95°C, rapid cooling to 35°C (20°C/second ramp rate), and gradual melting to 85°C at a ramp rate of 0.3°C/sec. A sharp decline in fluorescence is evident at ~51°C in the wild-type sequence (dotted line) and at ~59°C in the oligonucleotide sequence containing the Factor V Leiden mutation (dashed line). The higher T_m in the mutant sequence is explained by the fact that the fluorescein-labeled oligonucleotide probe is perfectly complementary to the Factor V Leiden mutation sequence. The F versus T graph for the negative DNA control (dots and dashes) is directly superimposed on that of the template-free (H_2O) control (solid line), and both show an expected gradual decrease in background fluorescence associated with increasing temperature. **B:** Derivative melting curves. **A** shows the derivative ($-dF/dT$ versus T) curves depicting the same data as in **B**. The derivative melting peaks are all oriented in positive scale and afford easier visualization of T_m s.

BSA. To assess the effect of pH on the fluorescence levels yielded by the dequenching chemistry, melting curves were performed in the presence of buffers ranging from pH 8.3 to 9.2.

The melting protocol consisted of denaturation at 95°C for 10 seconds, a rapid ramp down to 35°C at a rate of 20°C/sec, annealing at 35°C for 30 seconds, and heating to 85°C at 0.3°C/second. Probe melting peak analysis was performed using derivative plots ($-dF/dT$ versus T) as previously described.¹⁵

PCR Amplification and Genotyping

To determine whether PCR amplification would be compatible with fluorescence-dequenching genotyping, we performed fluorescence-dequenching PCR analysis using Factor V,¹⁶ MTHFR and human β -globin locus specific primers (Table 2). The design of the specific probes for each target is illustrated in Figure 3A (Factor V), Figure 4A (MTHFR), and Figure 5A (β -globin). Oligonucleotides were obtained from Genset Corporation (La Jolla, CA) and Operon Technologies.

Fluorescence-dequenching PCR for the Factor V Leiden and MTHFR loci was performed using the LightCycler. Fifty

Figure 2. Derivative melting curves from an oligonucleotide model system corresponding to a segment of the Factor V gene (wild-type) showing the relationship between fluorescence dequenching and pH. The buffer mixture and melting protocol used for this experiment are described in the Methods section. There is a progressive rise in fluorescence levels with increasing alkalinity up to pH 9.2.

Figure 3. Genotyping of the Factor V Leiden mutation by single-fluorophore dequenching. **A:** Probe design for single-fluorophore dequenching format. An 18-bp oligonucleotide probe complementary to the Factor V Leiden mutation sequence (G1691A) is labeled at the 3' end with fluorescein (F). The target sequence as depicted in this figure corresponds to the wild-type. The bolded base represents the substitution corresponding to the G1691A mutation. The fluorescein label is directly conjugated to a guanine base which leads to its quenching. Hybridization of the oligonucleotide probe to the cognate Factor V sequence leads to diminution of the quenching effect, and hence an augmentation of the fluorescence. **B:** Single-fluorophore dequenching melting peaks. Derivative melting curves reveal the highest T_m (~58°C) for the mutant allele (**dashed line**) to which the probe is perfectly complementary, and a lower T_m (~50°C) for the wild-type allele (**dotted line**) which has one mismatch (A-C). The heterozygote (**dots and dashes**) has two peaks at 58°C and 59°C, corresponding to the mutant and wild-type alleles respectively. The **solid line** represents the "no template" (H_2O) control, and displays no melting peak.

nanograms of DNA was amplified in a 10 μ l reaction containing 0.4 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), buffer (containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, at pH 9.2), 250 mg/ml BSA, 0.2 mmol/L each dNTP (dATP, dCTP, dGTP, dTTP), 3.0 mmol/L $MgCl_2$, 0.5 μ mol/L of each primer, and the fluorescein-labeled probe at 0.1 μ mol/L. The amplification protocol entailed an initial incubation at 95°C for 30 seconds followed by 45 cycles of denaturation (20°C/second ramp rate to 95°C for 0 seconds), annealing (20°C/second ramp rate to 50°C for 10 seconds), and extension (2°C/second ramp rate to 72°C for 10 seconds). After amplification, the products were cooled to 35°C and heated to 85°C at a rate of 0.3°C/second. Probe melting peak analysis was performed using $-dF/dT$ versus T plots. Fluorescence-dequenching PCR for the human β -globin locus was performed using the same protocol listed above, with the exception that the annealing temperature was 55°C.

Software

LightCycler software version 3.3 was used for all analyses. For dequenching melting curve analysis, fluorescence was detected in channel 1 with the gains set at 3.

Figure 4. Genotyping of the MTHFR C677T mutation by single-fluorophore dequenching. **A:** Probe design for single-fluorophore dequenching format. A 20-bp oligonucleotide probe complementary to the MTHFR mutation sequence (C677T) is labeled at the 5' end with fluorescein (F) blocked at the 3' end with a phosphate moiety. The target sequence as depicted in this figure corresponds to the wild-type. The bolded base represents the substitution corresponding to the C677T mutation. The fluorescein label is directly conjugated to a guanine base which leads to its quenching. Hybridization of the oligonucleotide probe to the cognate MTHFR sequence leads to diminution of the quenching effect, and hence an augmentation of the fluorescence. **B:** Single-fluorophore dequenching melting peaks. Derivative melting curves reveal the highest T_m (~68°C) for the mutant allele (**dashed line**) to which the probe is perfectly complementary, and a lower T_m (~65°C) for the wild-type allele (**dotted line**), which has one mismatch (T-G). The heterozygote (**dots and dashes**) has a broad peak with maximal height at 66°C. The **solid line** represents the "no template" (H_2O) control, and displays no melting peak.

DNA Sequencing

Automated DNA sequencing of PCR products was performed using dideoxynucleotide termination chemistry and the Applied Biosystems 3100 Genetic Analyzer.

Results

Artificial Templates

The single-fluorophore dequenching reactions are depicted as peaks in the positive scale in the $-dF/dT$ versus T graphs (Figure 1). For the Factor V Leiden mutation, the homozygous wild-type yielded a melting peak with T_m at $51.4 \pm 0.1^\circ\text{C}$ and the homozygous mutant yielded a melting peak with T_m $59.4 \pm 0.1^\circ\text{C}$. For MTHFR, the homozygous wild-type yielded a melting peak with T_m at 64.9°C and the homozygous mutant yielded a melting peak with T_m at 67.0°C (data not shown). These experiments demonstrated the ability of the single-fluorophore dequenching method to discriminate between DNA sequences differing by single nucleotide substitutions.

Figure 5. Genotyping of hemoglobin S and C mutations by single-fluorophore dequenching. **A:** Probe design for single-fluorophore dequenching format. A 21-bp oligonucleotide probe complementary to the human β -globin S sequence is labeled at the 3' end with fluorescein (F). The target sequence as depicted in this figure corresponds to the wild-type, read anti-parallel. The bolded bases represent the substitutions corresponding to the HbS and HbC mutations, respectively. The fluorescein label is directly conjugated to a guanine base which leads to its quenching. Hybridization of the oligonucleotide probe to the cognate β -globin sequence leads to diminution of the quenching effect, and hence an augmentation of the fluorescence. **B:** Single-fluorophore dequenching format. Derivative melting curves reveal the highest T_m ($\sim 64^\circ\text{C}$) for the HbS allele (**close dots**) to which the probe is perfectly complementary, and a lower T_m ($\sim 59^\circ\text{C}$) for the wild-type allele (**spaced dots**) which has only one mismatch (A:A). Predictably, the lowest T_m ($\sim 55^\circ\text{C}$) is observed for the HbC allele (**dashed line**) that has 2 mismatches (C:A, A:A). The HbSC compound heterozygote (**dots and dashes**) has two peaks at 64°C and 55°C , corresponding to the HbS and HbC alleles respectively. The **solid line** represents the "no template" (H_2O) control, and displays no melting peak.

pH Curve

The magnitude of change in fluorescence observed for the dequenching assays was dependent on the pH of the reaction solution. The results for the Factor V wild-type melting peaks in solutions of pH 8.3, 8.6, 8.9, and 9.2 are shown in Figure 2. The assays were optimally performed at pH 9.2, which was nonetheless compatible with PCR amplification as shown below.

Dequenching PCR and Genotyping

Factor V Locus

The single-fluorophore dequenching reactions are depicted as peaks in the positive scale in the $-dF/dT$ versus T graphs (Figure 3B). The G1691A homozygous samples yielded a probe melting peak with T_m at $58.6 \pm 0.9^\circ\text{C}$ and the wild-type samples yielded a melting peak with T_m at $50.3 \pm 0.7^\circ\text{C}$. The heterozygous samples yielded two melting peaks; each corresponding to the respective peaks observed for the mutant and wild-type homozy-

gotes. Using melting profiles obtained from the single probe dequenching experiments, we successfully genotyped 100 of 100 of the cases examined for mutations in wild-type ($n = 65$), heterozygous mutant ($n = 23$), and homozygous mutant ($n = 12$) samples. There was 100% concordance between the results of the single-fluorophore dequenching and a previously described dual-probe based method.⁶ (Table 3).

MTHFR Locus

The single-fluorophore dequenching reactions are depicted as peaks in the positive scale in the $-dF/dT$ versus T graphs (Figure 4B). The C677T homozygous samples yielded a probe melting peak with T_m at $67.8 \pm 0.4^\circ\text{C}$ and the wild-type samples yielded a melting peak with T_m at $64.9 \pm 0.6^\circ\text{C}$. The heterozygous samples yielded a broad melting peak spanning the respective T_m s observed for the mutant and wild-type homozygotes with a T_m at $66.3 \pm 0.8^\circ\text{C}$. Using melting profiles obtained from the single probe dequenching experiments, we successfully genotyped 20 of 20 of the cases examined for mutations in wild-type ($n = 10$), heterozygous mutant ($n = 5$), and homozygous mutant ($n = 5$) samples. There was 100% concordance between the results of the single-fluorophore dequenching and a previously described dual probe based method⁸ (Table 3).

Human β -globin Locus

The single-fluorophore dequenching reactions are depicted as peaks in the positive scale in the $-dF/dT$ versus T graphs (Figure 5B). The HbSS samples yielded a probe melting peak with T_m at $64.2 \pm 1.0^\circ\text{C}$, the wild-type samples yielded a melting peak with T_m at $60.2 \pm 2.2^\circ\text{C}$, and the HbCC samples yielded a melting peak with T_m at $56.5 \pm 0.6^\circ\text{C}$. The HbSC samples yielded two melting peaks; each corresponding to the respective peaks observed for the HbSS and HbCC homozygotes. Using melting profiles obtained from the single-probe dequenching experiments, we successfully genotyped 52 of 52 of the cases examined for mutations in the HbAA ($n = 37$), HbSS ($n = 5$), HbCC ($n = 3$), and HbSC ($n = 7$). There was 100% concordance between the results of the single-fluorophore dequenching and DNA sequencing or HPLC-based assays (Table 3).

Discussion

The fluorescence phenomena that have been most commonly exploited for the homogeneous detection of nucleic acids involve fluorescence resonance energy transfer (FRET) and/or quenching.¹⁷ FRET is a quantum phenomenon that occurs when excitation energy is transferred from a donor to an acceptor fluorophore with overlapping emission and absorption spectra.^{18,19} Energy transfer occurs through non-radiative dipole-dipole interactions, and has been used as a spectroscopic measure

Table 3. Accuracy of Single-Probe Dequenching for Genotypic Analysis of Factor V, MTHFR, and β -Globin Gene Loci

Target	Genotype	Number of samples	Scoring accuracy*
Factor V Leiden	Wild-type	65	100%
	Heterozygous (G1691A)	23	100%
	Homozygous mutant (G1691A)	12	100%
MTHFR	Wild-type	10	100%
	Heterozygous (C677T)	5	100%
	Homozygous mutant (C677T)	5	100%
β -Globin	HbAA	37	100%
	HbSS	5	100%
	HbCC	3	100%
	HbSC	7	100%

*Scoring accuracy was determined by comparison of results of genotyping using DNA sequencing, high-performance liquid chromatography and for dual hybridization probe-based systems.

of molecular distances ranging from 1 to 10 nm.^{18,20,21} When FRET occurs, the fluorescence intensity, half-life, and quantum yield of the donor decrease, while the fluorescence intensity of the acceptor increases.²² On the other hand, fluorescence quenching results in reduction of the quantum yield of a fluorophore without altering the wavelength of the emitted fluorescence spectrum.²³ Quenching may involve energy transfer, dimer formation between closely situated fluorophores, transient excited-state interactions, collisional quenching, or formation of non-fluorescent ground state species.²⁴

Several fluorescence properties such as intensity, half-life, and emission spectrum are altered as a consequence of hybridization.^{25,26} For instance, fluorescence quenching occurs during probe hybridization when fluorescein^{15,27} or BODIPY-FL²⁸ is brought in close approximation to deoxyguanosine nucleotides. The probe can be labeled on either the 3' or the 5' end with similar quenching efficiency. Maximum quenching efficiency is achieved when the probe-target interaction is such that a G is present at the first overhang position on the target strand. Additional neighboring Gs on the target strand increase quenching incrementally, but a G in the first overhang position is most essential.²⁷

The phenomenon of dequenching has recently been described and exploited for the analysis of a number of biological parameters. In this regard, dequenching has been used to measure the dilution of liposome-entrapped fluorophore caused by changes in membrane permeability or membrane fusion.^{29,30} Dequenching of a self-quenching fluorogenic probe labeled with octadecylrhodamine and specific for a hydrophobic binding pocket of the activator protein that is mutated in G(M2) gangliosidosis, has been used to characterize the oligosaccharide-binding specificity of the activator protein.³¹ With regard to nucleic acids, dequenching has been used for the analysis of RNA degradation *in vitro* and *in vivo*.³² In addition, Lee and colleagues²⁵ have used fluorescence dequenching for kinetic studies of restriction endonucleases. The dequenching-based assay provided an easy and rapid method for acquiring detailed data density necessary for precise kinetic studies. However, dequenching-based strategies have not been used for the

detection of single nucleotide polymorphisms or genotyping.

In this study, we show that the phenomenon of hybridization-induced fluorescence dequenching can be used for nucleic acid detection and genotyping. In our experiments, we directly conjugated fluorescein to a guanosine base at either the 5' or the 3' end of an oligonucleotide probe complementary to the target of interest. The close proximity of the fluorescein to the guanine base typically results in quenching of fluorescence.^{15,27} This deoxyguanosine-mediated quenching effect has previously been considered problematic for the design of FRET-based probes³³ and in DNA sequencing.^{34,35} Nevertheless, hybridization of the fluorescein-labeled probe to the unlabeled complementary strand resulted in dequenching of the fluorophore, and an increase in fluorescence was observed. Although the signal generated by the dequenching approach was weaker than that obtained with the dual probe approach, our studies show that it is robust enough for routine genotyping of clinical samples. In all cases PCR amplification did not exceed 45 cycles, and consequently contamination was not a problem. Interestingly, we noted that the intensity of the fluorescence was influenced by the pH of the reaction solution, with increasing pH favoring increased fluorescence above background up to pH 9.2, at which the analyses were optimally performed and compatible with PCR amplification.

In conclusion, our studies show that the dequenching format of single fluorophore-based reporting systems for nucleic acid detection and PCR monitoring combine the advantages of simplicity, ease of design, and superior specificity to that provided by non-specific DNA binding dyes or intercalators. Further, single-labeled fluorophore based systems obviate the requirement for inclusion of an additional fluorophore without sacrificing specificity of product detection. The fluorescence of single-labeled probes is also reversible and depends only on hybridization of the probe to the target, allowing study of the melting characteristics of the probe from the target, thereby facilitating genotyping by Tm. We anticipate that the single-fluorophore dequenching format will be adapted for a diverse number of applications in molecular research and diagnostics.

Acknowledgments

We thank Dr. Christine Litwin of the Section of Clinical Immunology, Microbiology and Virology, Department of Pathology, University of Utah School of Medicine, and Dorothy Hussey at ARUP Laboratories, Inc. for provision of clinical samples.

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