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Nucleic Acid Analysis Using an Expanded Genetic Alphabet to Quench Fluorescence

Christopher B. Sherrill, David J. Marshall, Michael J. Moser, Christine A. Larsen, Lygia Daudé-Snow, and James R. Prudent*

Contribution from Eragen Biosciences, Inc., 918 Deming Way, Madison, Wisconsin 53717

Received December 5, 2003; E-mail: jprudent@eragen.com

Abstract: Organic chemistry has made possible the synthesis of molecules that expand on Nature's genetic alphabet. Using the previously described nonstandard DNA base pair constructed from isoguanine and 5-methylisocytosine, we report a highly specific and sensitive method that allows for the fast and specific quantitation of genetic sequences in a closed tube format. During PCR amplification, enzymatic site-specific incorporation of a quencher covalently linked to isoguanine allows for the simultaneous detection and identification of multiple targets. The specificity of method is then established by analysis of thermal denaturation or melting of the amplicons. The appropriate functions of all reactions are further verified by incorporation of an independent target into the reaction mixture. We report that the method is sensitive down to the single copy level, and specificity is demonstrated by multiplexed end-point genotypic analysis of four targets simultaneously using four separate fluorescent reporters. The method is general enough for quantitative and qualitative analysis of both RNA and DNA using previously developed primer sets. Though the method described employs the commonly used PCR, the enzymatic incorporation of reporter groups into DNA site-specifically should find broad utility throughout molecular biology.

Introduction

Since the publication of the polymerase chain reaction (PCR) in 1987, applications involving this technology have revolutionized molecular medicin.[1] Advanced technologies such as real-time PCR, facilitated by developments in fluorogenic molecule chemistries, are rapidly replacing traditional post-amplification analysis methods. Because of the inherent benefits of real-time PCR technology, namely, enhancements in specificity, robustness, and quantitation potential, it is rapidly becoming the preferred approach for nucleic acid based tests in diagnostic laboratories. In an attempt to simplify real-time analysis, we employed an expanded genetic alphabet.

It was previously observed that the DNA alphabet need not be limited to the four standard nucleotides known in Nature.[2,3] Rather, 12 nucleobases forming six base pairs joined by mutually exclusive hydrogen bonding patterns might be possible within the geometry of the Watson−Crick base pair. In natural DNA, two complementary strands are joined by a sequence of Watson−Crick base pairs.[4,5] These obey two rules of complementarity: size (large purines pair with smaller pyrimidines) and hydrogen bonding (hydrogen bond donors from one nucleobase pair with hydrogen bond acceptors from the other). The former is necessary to permit the structure that underlies enzyme recognition. The latter achieves the specificity that gives rise to the simple rules for base pairing ("A pairs with T, G pairs with C") that underlie genetics and molecular biology. No other class of natural products has reactivity that obeys such simple rules. Nor is it obvious how one designs a class of chemical substances that does so much so simply. The synthetic base pair formed by isoguanine (iG) and 5′-methylisocytosine (iC) has been successfully employed for both molecular recognition[6−8] and for site-specific enzymatic incorporation.[2,9,10] We have covalently coupled a diverse list of reporter groups to 2′-deoxyisoguanosine triphosphate (diGTP), through a modification of the *N*-6. The list includes dabcyl, a smal-molecule fluorescent quencher used in this report to quench a wide range of fluorescent molecules attached to DNA (Figure 1). We have

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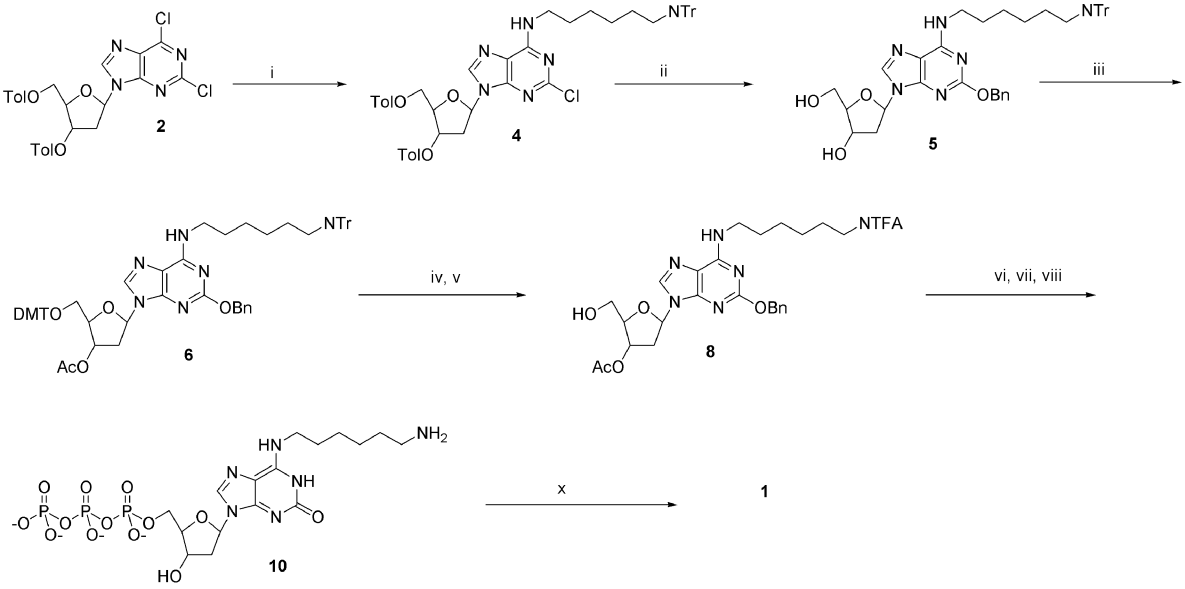
Scheme 1. Synthesis of Dabcyl-diGTPa



[a] Key: (i) **3**, DMF; (ii) NaH, BnOH, DMF; (iii) DMT-Cl, Ac_2O, pyridine; (iv) MeOH, iodine; (v) TFA_2O, pyridine; (vi) $POCl_3$, $(Bu)_3N$ pyrophosphate, MeCN; (vii) NH_4COO, Pd/C, MeOH; (viii) NH_4OH; (ix) Dabcyl-OSu, DMF, H_2O.

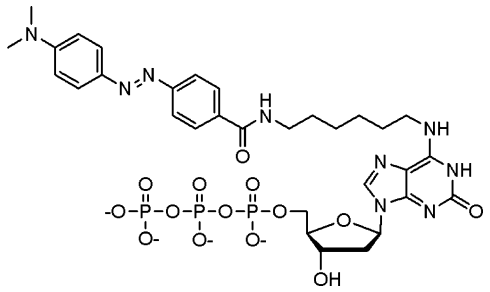


Figure 1. Structure of dabcyl-diGTP (**1**).

found that these labeled diGTP molecules make excellent substrates for a number of thermal stable polymerases incorporating efficiently opposite iC.

The synthesis of a modified diGTP has not been reported previously. Synthesis of a similar compound (modified iGTP) has been described, yet the strategy described here is notably different (Scheme 1).[11] Exploiting the higher reactivity of the 6 position, the monoprotected diaminohexane is introduced in the first step to the known ditoluoyldichlorodeoxyriboside (**2**).[12] The benzyloxy group is then introduced at the 2 position, simultaneously removing the sugar protecting groups, to provide **5**. Differential protection of the 5′ and 3′ hydroxyls is achieved in one step as the dimethoxytrityl ether and the acetyl ester, respectively. To effect the deprotection of the 5′-hydroxyl and the amine of the tether, without also cleaving the rather acid sensitive gylcosyl bond, an unusual method utilizing HI generated in situ was employed; the tethered amine was then reprotected as the TFA-amide to provide **8**.[13] After introduction of the triphosphate in a two-step process using $POCl_3$ followed by pyrophosphate, the benzyl protection of the 2-oxo group and the acetyl protecting groups were removed to provide **11**, which was then highly purified by HPLC. This served as a convenient source of starting material to introduce a variety of reporter groups into the diGTP structure for systematic enzymology and assay development experiments. In this case, dabcyl was introduced through its succinimidyl ester and HPLC purified to provide the target compound, dabcyl-diGTP (**1**).

In an attempt to simplify real-time PCR analysis, we postulated that the accumulation of PCR amplicons could be monitored by the incorporation of a quencher close to a fluorophore-labeled primer. To do this efficiently, we employed iC:iG base pairing to site specifically place the quencher in direct proximity to the fluorophore. A nuclease minus *Thermus aquaticus* polymerase was used to carry out the PCR and incorporate the dabcyl-diGTP opposite the iC (Figure 2A). To minimize the distance between the quencher and the fluorophore, one primer flanking the target was labeled at the 5′-end with fluorescein adjacent to a single iC nucleotide. In this model, the quenching effect of the dabcyl-diGTP incorporated into the amplicons could be used to monitor the progress of the PCR. Not only was the fluorescence of the amplicon quenched, but also the quenching was directly proportional to the level of product formation (Figure 2B). This is the first report demonstrating efficient incorporation of a labeled diGTP into a DNA duplex by a DNA polymerase.

To determine the range of quenching using this approach, a number of reporter groups were tested and the percent quenching determined (Table 1). The data indicated that a wide spectral range of fluorescent dyes can be quenched, most likely due to the close proximity of the dye-quencher pair. Based on the diameter of the double helix and the length of the linkers used, we estimate that the average distance between the pair ranges from 0 to 50 Å Quenching efficiency was variable, as expected.

To examine the method's ability to quantify naturally occurring nucleic acid sequences, the HIV *gag* gene was used as a model system. DNA and RNA sequences matching a region found within the nucleocapsid portion of the HIV gag gene were synthesized and 10-fold dilutions were generated. Previously

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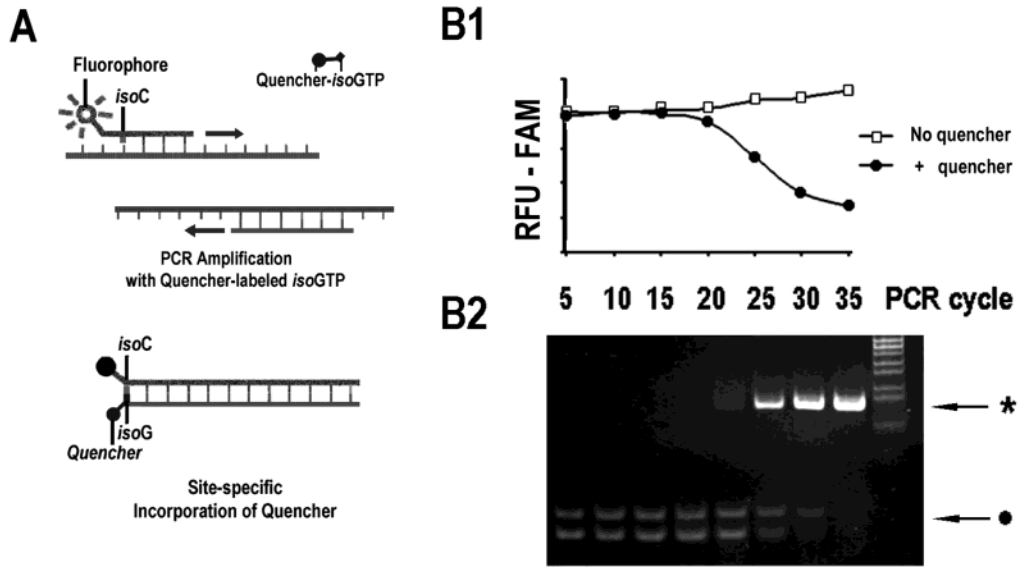

Figure 2. Principles of site-specific incorporation of a quencher during amplification. (A) Site-specific quencher incorporation: A target is amplified containing a single isocytosine nucleotide and a 5/6-carboxy fluorescein (FAM) label. An unlabeled standard base reverse primer is used. Amplification is performed in the presence of dabcyl-diGTP. Site-specific incorporation places the quencher in close proximity to the fluorophore. (B1) PCR reactions were performed in the presence (filled circles) or absence (open squares) of dabcyl-diGTP. Based on model design, the only position where diGTP was allowed to incorporate was at the 3′ end of the reverse primer. After every fifth round of cycling a 5 μL aliquot was removed and analyzed using a Cytofluor 4000 flourescence plate reader. (B2) The 5 μL aliquots were then used for PAGE analysis. The gel was stained with EtBr and scanned for fluorescence. Fluorescence imaging detects PCR primers (•) and the PCR product (*) from reactions containing dabcyl-diGTP.

Table 1. Percent Quenching of a List of Dyes by Dabcyl Using a Model Experiment Mimicking the Approach Described[a]

fluorophore	% quenching	emission peak (nm)
FAM	62	518
TET	53	536
HEX	71	554
ROX	64	606
LC640	48	640
Cy5	35	667

[a] FAM = fluorescein, TET = tetrachlorofluorescein, HEX = hexachlorofluorescein, ROX = rhodamine, and Cy5 = cyanine 5.

designed PCR primers SK38 and SK39 were constructed which amplify a 115 bp region of the HIV *gag* gene.[14] To detect both RNA and DNA, Moloney murine leukemia virus reverse transcriptase (M-MuLV-RT) was included while in its absence only DNA targets were amplified. We observed that the target number added to each reaction correlated to the cycle number where signal decrease crossed a fixed fluorescent threshold (Figure 3, parts A1 and B1). These findings indicate that this approach allows for a linear quantitation of both RNA and DNA targets (Figure 3, parts A3 and B3).

Since the amplicon contains quencher and fluorophore on opposite strands of the DNA duplex, slow heating and optical analyses provide valuable product melting temperature (T_m) information. The correct product possesses a unique T_m that is dependent on base composition.[15] Melt profiles from target specific products typically differ from those of target independent products.[16] For the HIV gag-related sequence experiments, the T_m appears to be approximately 76.5 °C (Figure 3, parts A2 and B2).

The capability of performing post-reaction melt analysis based on the thermodynamics also enables genotypic end-point analysis. To determine the efficiency of end-point analysis and to demonstrate the multiplexed ability of this method, we chose to simultaneously analyze two single base changes within the HFE gene (H63D and C282Y). These mutations have been shown to be linked to the majority of hemochromatosis disease cases, a common autosomal recessive disorder of iron metabolism characterized by increased iron absorption.[17] We first designed PCR primer sets for the H63D and the C282Y sites (four forward and two reverse). Each forward primer had a specific fluorescent dye attached to its 5′ end (FAM, HEX, ROX, or LC640) and was made specific for either the wild-type or mutation sequence by changing the 3′-end base. The reverse primers were region specific and designed to create amplicons that would have differing thermodynamic properties. The amplicon generated for H63D had a melting temperature of approximately 83 °C, and the C282Y amplicon had a melting temperature of approximately 86 °C. The reaction conditions were identical to those used for analyzing the HIV-specific DNA, but this time there were six primers targeting to regions in each reaction. Human genomic DNAs with known HFE mutations were used to test the system. When no DNA was added, the post-reaction melt results indicate that no amplicons were generated. When specific DNA was added, the results indicate that correct genotypes could be determined after the cycling was complete (Figure 4). The results were tabulated and the findings indicate that this approach can be broadly used to analyze genotypes (Table 2).

In this paper, we introduce a novel approach for the detection of DNA or RNA targets. Using an optimized reagent mixture

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Table 2. Fourplex Genotype Determination of Human Genomic DNA Samples[a]

sample	NTC	NA14897	NA135891	NA16000	NA14640	NA14641	
H63 genotype	N/A	WT	H63D Mut.	H63D Het.	WT	H63D Het.	
C282 genotype	N/A	WT	WTjt	WT	C282Y Mut.	C282Y Het.	Dye
C282C	N/A	86.0	86.0	86.0	N/A	85.5	FAM
C282Y	N/A	N/A	N/A	N/A	85.5	85.5	HEX
H63D	N/A	N/A	83.5	84.0	N/A	83.5	ROX
H63H	N/A	83.0	N/A	83.5	83.5	83.5	LC640

[a] T_m and genotypes were assigned on the basis of the following criteria: FAM T_m from 85.5 to 86.0 °C indicates the presence of HFE C282C wild-type (WT) allele, HEX T_m from 85.5 to 86.0 °C indicates the presence of C282Y allele, ROX T_m from 83.5 to 84.0 °C indicates the presence of HFE H63D allele, LC640 T_m from 83.5 to 84.0 °C indicates the presence of HFE H63H (WT) allele; and dRFU greater than 30% of maximum dRFU for all samples. All genotype assignments are 100% concordant with previously determined HFE mutation status (Coriell Cell Repositories, NJ).

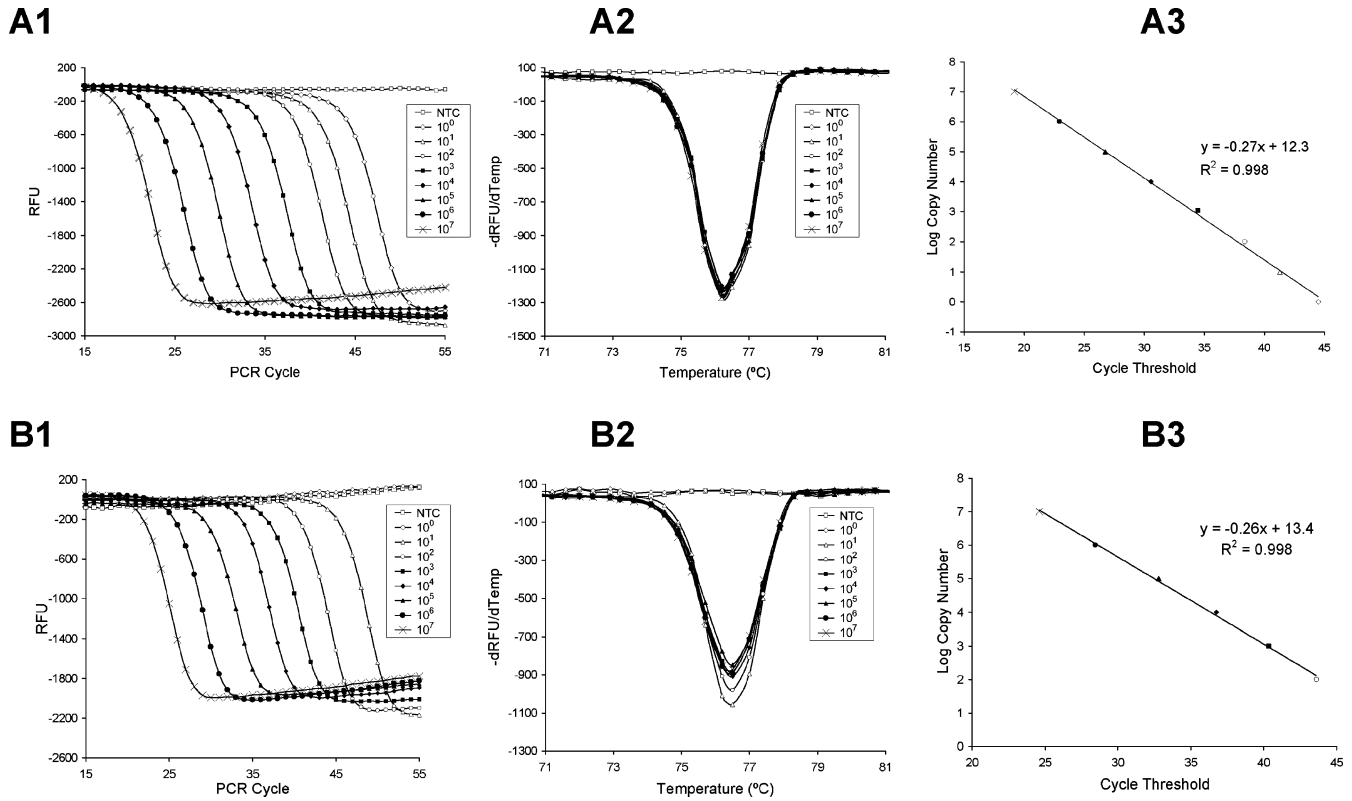

Figure 3. DNA (A) and RNA (B) quantitation and melt analysis. Ten-fold dilution series of the targets from 1 to 10^7 copies of DNA (A) or RNA (B) were quantified using 55 cycles of PCR or single-tube RT-PCR reaction (NTC = no target control). Fluorescence signals for each target concentration over the course of the reaction (A1 and B1). Negative first-derivative analyses of post-reaction thermodynamic melt curve ($-$dRFU/dT) versus temperature (in °C) (A2 and B2). Third standard curve of log copy number versus cycle threshold with equation, R^2, and linear fit (A3 and B3).

containing a modified nonstandard base to enzymatically incorporate a quencher opposite a variety of fluorophores, fluorescent change can be monitored in real-time to specifically quantify nucleic acids. Unlike all other real-time technologies, the incorporation results in a decrease in signal that is proportional to amplicon formation. This decrease can be analyzed using developed software to obtain a standard quantitation curve, similar to that of other methodologies that use an increase in signal. Multiple targets can be analyzed in a single reaction by simply using multiple fluorescent dye substitutions. We also demonstrated that previously designed primers can be utilized in our approach and believe that the method can be applied to most any nucleic acid target.

There are many real-time PCR technologies currently available with TaqMan, Molecular Beacons, and hybridization probes being the three most widely used.[18−21] However, these systems are somewhat difficult to design since they share an indirect detection mechanism: hybridization of a probe to a PCR product. Probe design may be complicated by the inherent fact the single-stranded DNA target forms intramolecular structures that interfere with probe binding.[22] Alternative systems that do not require probes include Amplifluor, LUX primers, and Scorpion primers.[23−25] Compared to these systems, our method does not require incorporation of hairpins in the primer design

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nor does it require special base sequence makeup near the 3′ ends. Probably the simplest method available for real-time PCR detection uses SYBR Green to detect PCR amplicon generation.[26] For SYBR Green as with our system, design is not an issue. With SYBR green the dye is simply added to the PCR reaction. The major drawback to SYBR green technology is that it cannot be multiplexed in real-time, though melt analysis has been shown to be capable of detecting product mixtures at the end of the reaction.

Since our method does not require an extra probe and specificity concerns may be raised, we compared our method to Taq-Man by designing a multiplexed assay for human GAPD and β-actin transcribed messages. There are over thirty pseudogenes for each target within the human genome. In the presence of 30,000 of copies of genomic DNA containing those psuedogenes, results from our method indicate that single copies of the specific transcripts could be detected.[27] This was similar to the results obtained using a highly optimized Taq-Man system. Today, most molecular biologists have experience designing PCR reactions and should have little difficulty using this novel real-time methodology.

The methodology described here offers many advantages over these other technologies. For example, since probes are not required, the method simplifies design issues and allows for rapid development. Also, the method directly measures amplicon generation permitting large dynamic range quantitation and postreaction melt analysis that is similar to results obtained by performing PAGE. Since multiplexing can be accomplished, multiple targets can be analyzed in a single reaction saving time and resources. In addition, internal controls can be implemented to guarantee proper reaction conditions.

We report here the use of four dyes simultaneously for end point genotypic analysis of genomic DNAs. To date, the maximum number of targets simultaneously queried in a single reaction using this method has been four using the dye combination fluorescein, hexachlorofluorescein, rhodamine and LC640. Previous reports using molecular beacons demonstrated that dabcyl could efficiently quench a large array of fluorophores.[28] It is conceivable that by using a combination of dyes and thermal melts, one could examine additional targets all within the same reaction. And as instrumentation and expanded base chemistry advance, it is likely that this approach will allow for even further real-time multiplexing capability.

More generally, for the first time we demonstrated that labeled diGTP can be efficiently synthesized and incorporated enzymatically. This will allow for broad use of such compounds in molecular biology. Any time a reporter group is required at a specific site within an amplicon, transcript or extension product, the use of an additional base pair may be helpful. Therefore, the use of expanded base methodology is surely not limited to PCR. Indeed, there are other detection methods that may benefit

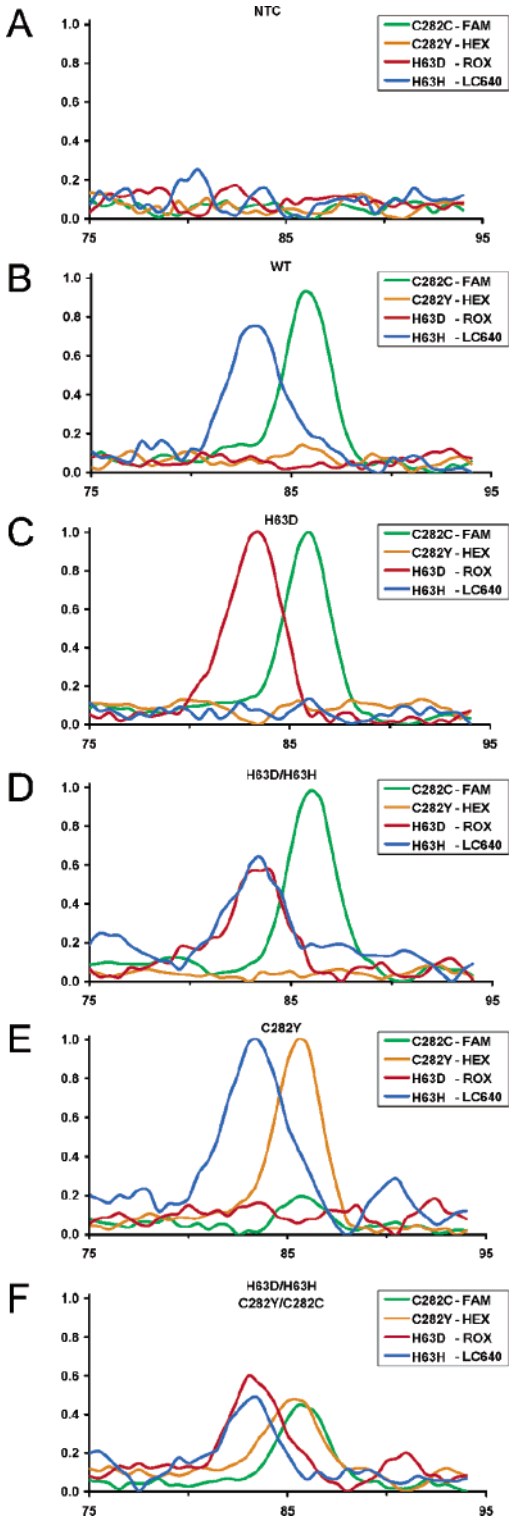

Figure 4. Four-color multiplex genotype determination by incorporation of dabcyl-diGTP. Five samples of human genomic DNA with known hemachromatosis (HFE) gene mutation status and a no target control (NTC) were analyzed using this method. Following amplification, PCR reaction products were melted from 75 to 95 °C in 0.5 °C increments, and four fluorescence channels were monitored at each increment. The first derivative of the rate of fluorescence change with temperature (dRFU/dTemp) is plotted vs temperature (in °C). HFE allele-specific primers were labeled with the following fluorophores: H63H, FAM (green line); C282Y, HEX (orange line); H63D, ROX (red line); C282C, LC640 (blue line). Coriell Cell Repository target designations used to obtain results for each graph were as follows: A, NTC; B, NA14897; C, NA135891; D, NA16000; E, NA14640; F, NA14641.

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from this chemistry. With such a fundamental paradigm shift, we envision that additional base pairs will change the way scientists build new testing systems and construct novel organisms.

Methods

Synthesis of Dabcyl-diGTP: 1-Tritylhexamethylenediamine (3). Hexamethylenediamine (10 equiv) was dried two times from pyridine and dissolved in pyridine (15 equiv). DMAP (0.1 equiv) was added and the reaction flask placed in an ice bath. Trityl chloride (1 equiv), dissolved in pyridine (15 equiv), was added dropwise over 2 h. It was stirred at room temperature for 4 h, MeOH (3.3 equiv) added, the reaction mixture concentrated, and the remaining residue extracted with aqueous $NaHCO_3$/ethyl acetate. The organic layer was washed twice with aqueous $NaHCO_3$ solution and dried and the solvent evaporated. The obtained product was used in the next step without further purification. Yield: 95%, sticky oil.

2-Chloro-6-(6tritylaminohexyl)aminopurine-2′-deoxy-3′,5′-ditoluylriboside (4). Compound **3** (1.3 equiv) was dried from DMF and dissolved in DMF (40 equiv). Diisopropylethylamine (3.9 equiv) and 2,6-dichloropurine-2′-deoxy-3′,5′-ditoluoylriboside (**2**) (1 equiv), dissolved in DMF (40 equiv), were added, and the mixture was stirred at room temperature for 3 h. It was concentrated by rotary evaporation, the residue extracted with aqueous $NaHCO_3$/ethyl acetate, the organic layer dried, and the solvent evaporated. The residue was triturated with ether twice and the obtained solid product used further after drying under vacuum without further purification.

2-Benzyloxy-6-(6tritylaminohexyl)aminopurine-2′-deoxyriboside (5). NaH (60% in mineral oil, 10 equiv) was slowly added to benzyl alcohol (67 equiv) with stirring at room temperature under argon. After the mixture was stirred for another 20 min at room temperature, compound **4** (1 equiv) was added. After the mixture was stirred for 20 min at room temperature, the reaction flask was transferred to a heated oil bath (100 °C). After 30 min, the reaction flask was cooled and the mixture added to ethyl acetate/$NaHCO_3$. The aqueous phase was extracted with ethyl acetate once more, and the pooled ethyl acetate layers were dried and concentrated by rotary evaporation. TLC: 4% MeOH/$CHCl_3$, $R_f = 0.19$. Product was purified by Et_3N-neutralized silica gel chromatography with 3% MeOH/$CHCl_3$ as the eluent. Yield: 71%.

2-Benzyloxy-6-(6-tritylaminohexyl)aminopurine-3′-*O*-acetyl-2′-deoxy-5′-*O*-*p*,*p*′-dimethoxytritylriboside (6). In a flame-dried flask, under argon, was dissolved compound **5** (1 equiv) in anhydrous pyridine (31 equiv). DMT chloride (0.6 equiv) was added and the mixture stirred for 30 min before a second addition of DMT chloride (0.6 equiv). Additional DMT chloride (0.2 equiv) was added as needed. TLC: 5% MeOH/$CHCl_3$, $R_f = 0.06$. When the DMT reaction was complete, acetic anhydride was added (2 equiv) and the reaction continued stirring 1 h. Additional acetic anhydride (2 equiv) was added as needed before addition of MeOH to quench excess reagent. TLC: 1.5% MeOH/$CHCl_3$, $R_f = 0.88$. The reaction mixture was concentrated and extracted (chloroform/5% $NaHCO_3$), and the pooled organic layers were dried and evaporated. Product was purified by Et_3N-neutralized silica gel chromatography with 0.3% MeOH/$CHCl_3$ as the eluent. Yield: 61%.

2-Benzyloxy-6-(6-aminohexyl)aminopurine-3′-*O*-acetyl-2′-deoxyriboside (7). Compound **6** (1 equiv) was added to a sealed-reaction flask containing a stir bar. A solution of iodine (0.9 equiv) in methanol (99% v/w) was added to the flask, and it was sealed. The flask was placed in an oil bath (60 °C) and stirred (8 h). The reaction was cooled to room temperature, and the volatiles were removed by rotary evaporation followed by high vacuum. Cleaved DMT and trityl protecting groups were removed from the product by trituration with diethyl ether. TLC: 2.5% MeOH/ $CHCl_3$, $R_f = 0.00$. Material was used in the next step without further purification.

2-Benzyloxy-6-(6-trifluoroacetamidohexyl)aminopurine-3′-*O*-acetyl-2′-deoxyriboside (8). Compound **7** was dissolved in anhydrous pyridine (117 equiv) in a flame-dried flask containing a stir bar under argon. Trifluoroacetic anhydride (2 equiv) was added and the reaction stirred until no starting material was detectable by TLC. Additional trifluoroacetic anhydride was added (1 equiv) as necessary. MeOH (5 equiv) was added before the reaction mixture was concentrated by rotary evaporation. The residue was dissolved in $CHCl_3$ and extracted with brine. The $CHCl_3$ was dried and evaporated, and the product was purified by silica gel chromatography with 1.5% MeOH/$CHCl_3$ as the eluent. TLC: 7.5% MeOH/ $CHCl_3$, $R_f = 0.53$. Yield: 40% from **6**.

2-Benzyloxy-6-(6-trifluoroacetamidohexyl)aminopurine-3′-*O*-acetyl-5′-triphosphoryl-2′-deoxyriboside (9). Freshly recrystallized imidazole (3.2 equiv) was dissolved in acetonitrile (15 equiv) under argon and chilled (0 °C). $POCl_3$ (1 equiv) and anhydrous triethylamine (3.2 equiv) were then added, and the mixture was stirred (0 °C, 0.5 h) before addition of a portion (1.3 equiv $POCl_3$) to **8**. This mixture was stirred (rt, 0.5 h) before addition of DMF (242 equiv) containing tributylammonium pyrophosphate (2 equiv). The reaction was then quenched (10% NH_4COO) 24 h later and lyophilized. Product was purified by anion-exchange chromatography (Dionex ProPac SAX-10) using 20% MeCN and a gradient of $(NH_4)_2CO_3$/20% MeCN. Collected product was repetitively lyophilized to remove excess salt. Yield: 20%, white solid.

6-(6-Aminohexyl)-5′-triphosphoryl-2′-deoxyisoguanosine (10). Compound **9** was dissolved in methanol (9 kequiv) before addition of Pd/C (10%, 0.02% w/v) and NH_4COO (7 equiv). The suspension was refluxed (1 h) before filtering off the catalyst and evaporating the solvent. The residue was then treated with ammonium hydroxide (30%, 3 kequiv, 3 h, room temperature). The product was purified by anion-exchange chromatography (Dionex ProPac SAX-10) using 20% MeCN and a gradient of $(NH_4)_2CO_3$/20% MeCN. Collected product was repetitively lyophilized to remove excess salt. Yield: 90%, white solid.

6-(*N*-(Dabcyl)-6-aminohexyl)-5′-triphosphoryl-2′-deoxyisoguanosine (1). To **10** (0.88 μmol, triethylammonium salt) in H_2O (40 μL) was added sodium borate buffer (10.5 μL, 1 M, pH 8.5) followed by DMF (216 μL) containing dabcyl *N*-hydroxysuccinimide ester (6 equiv). The reaction proceeded (8 h, 55 °C) before it was diluted with 20% MeCN and the product purified by anion-exchange chromatography (Dionex ProPac SAX-10) using 20% MeCN and a gradient of $(NH_4)_2CO_3$/20% MeCN. Collected product was repetitively lyophilized to remove excess salt. Yield: 50%.

Synthesis of Oligonucleotides. PCR oligonucleotides were manufactured with a 48-column DNA Synthesizer (Northwest Engineering, Alameda, CA), using standard β-cyanoethyl phosphoramidite chemistry. The isoC phosphoramidites, N^2-(dimethylamino)methylidene-5′-*O*-dimethoxytrityl-5-methyl-2′-deoxyisocytidine and 3′-*O*-cyanoethoxydiisopropylphosphoramidite (Glen Research, Sterling, VA), were coupled and deprotected under the conditions used for the standard base phosphoramidites. Postsynthesis workup consisted of ammonium hydroxide deprotection followed by ethanol precipitation. When required, oligonucleotides were further purified by anion exchange HPLC and desalting using C-18 resin.

Fluorescence Quenching during PCR. A PCR reaction was performed to demonstrate fluorescence quenching by site-specific incorporation in PCR. PCR conditions: 0.2 μM first primer; 0.2 μM second primer A; 0.4 pM template nucleic acid; 50 μM each dATP, dGTP, dTTP, and dCTP; 10 mM Tris-HCl pH 8; 0.1% BSA; 0.1% Triton X-100; 0.1 μg/μLdegraded herring sperm DNA; 40 mM potassium acetate; 2 mM magnesium chloride; 1 unit of Klentaq DNA polymerase (Ab Peptides, St. Louis, MO); and 0 or 3.0 μM dabcyl-diGTP in a 25 μL reaction volume. Reactions were analyzed by a Cytofluor 4000 fluorescence plate reader (485 nm excitation/530 nm emission) and by gel electrophoresis. PCR reactions were analyzed by 10% native polyacrylamide gel electrophoresis and fluorescence imaging using a Typhoon fluorescence scanner (Molecular Dynamics, Sunnyvale, CA).

Percent Quenching of Fluorescent Dyes. Synthetic DNA templates were amplified in 25 rounds of PCR as described above. Target concentration was 20 pM and the dye-labeled PCR primers contained a single 5′ iC adjacent to various fluorophores including 6-carboxy fluorescein (FAM), tetrachlorofluorscein (TET), hexachlorofluorescein (HEX), rhodamine (ROX)μ, LightCycler 640 (LC640) (Roche), and cyanine 5 (Cy5). The FAM, TET, HEX, and Cy5 fluorophores were coupled to dye-labeled PCR primers during oligonucleotide synthesis by phosphoramidite addition, while LC640 and ROX were added postsynthetically by succinimidyl ester reaction with a 5′ amine added as a phosphoramidite during synthesis. All primers were purified by denaturing anion-exchange HPLC prior to use. PCR cycling parameters were 5 s at 95 °C, 5 s at 55 °C; 20 s at72 °C with optics on. Decrease in PCR reaction fluorescence was monitored in real-time using the iCycler IQ (BioRad). The percent quenching was calculated as the ((initial reaction fluorescence − final reaction fluorescence)/ initial reaction fluorescence) × 100.

Real-Time PCR and RT-PCR by Site-Specific Incorporation of Dabcyl-diGTP. Previously described PCR primers SK38 and SK39 which amplify a 115 bp region of the HIV gag gene were modified to allow fluorescence monitoring and incorporation of dabcyl-diGTP.[14] Primer length was reduced by 5′ truncation to achieve a predicted T_m of 60 °C. The reverse primer used to transcribe the RNA transcript into cDNA was all standard deoxynucleotides while the forward primer contained a single 5′-iC adjacent to a terminal FAM fluorophore. DNA plasmid containing HIV gag gene isolated from pNL4-3 was used as a template for PCR and for in vitro transcription to RNA template by T7 RNA polymerase. PCR and RT-PCR reactions were performed using from zero to 1×10^7 copies of DNA and RNA target as estimated by absorbance at 260 nm. PCR conditions were 1× ISOlution buffer (EraGen, Madison, WI) with PCR primers at a concentration of 300 nM, dithiothreitol added at 5 mM, and Titanium Taq DNA polymerase (Clontech, CA) at the manufacturer's recommended concentration. PCR cycling parameters were 2 min denature at 95 °C followed by 50 cycles of 5 s at 95 °C, 5 s at 55 °C; 20 s at 72 °C with optical read on the Prism 7700 (Applied Biosystems Inc., Foster City, CA) real-time thermal cycler. A thermal melt with optical read from 60 to 95 °C was performed directly following the last 72 °C step of thermal cycling. For RNA templates, M-MuLV RT (Promega, Madison, WI) was added at 0.5 units/μL, and an initial 5 min incubation at 50 °C was performed prior to PCR amplification to reverse transcribe RNA to DNA. Omission of M-MuLV RT eliminates signal decrease for RNA templates (data not shown). Raw FAM component fluorescence data was exported from SDS 1.9 (Applied Biosystems, Inc.) software and analyzed with the analysis software discussed below.

Multiplex Genotype Determination by End-Point Thermal Melt. Primers were designed for simultaneous multiplex genotyping of two mutations in the human hemochromatosis (HFE) gene.[17] The following primer sequences were used in 5′ to 3′ order: LC640-NH2-TX-ACACGGCGACTCTCATG, ROX-NH2-TXACACGGCGACTCT-CATC, TTGTTTGAAGCTTTGGGCTAC, FAM-TXGGGAAGAG-CAGAGATATACGTG, HEX-TXGGGAAGAGCAGAGATATACGTA, TCAGCTCCTGGCTCTCA. PCR conditions were similar to those used to analyze the HIV gag gene with the following modifications: PCR cycling parameters were 2 min denature at 95 °C followed by 45 cycles of 1 s at 95 °C, 1 s at 58 °C, 24 s at72 °C. Primers were at 200 nM. Ten nanograms of human genomic DNA templates containing known HFE mutations (Coriell Cell Repositories, Haddon, NJ) were used as targets for amplification. Following amplification, the reactions were subjected to a thermal melt from 75 to 95 °C in 0.5 °C increments on the iCycler iQ (BioRad, Hercules, CA), with optical read for FAM, HEX, ROX, and LC640 fluorophores at each 0.5 °C step. Negative first derivative of the rate of change in relative fluorescence (RFU) vs temperature (°C) ($-$dRFU/dT) was exported from the iCycler iQ software. The minimum of dRFU/dT was set to zero for each fluorophore. The data were then normalized by dividing by the maximum dRFU/dT value (set dRFU/dT maximum = 1.0). The T_m value for each quenched, fluorophore-labeled PCR product was identified by the temperature at which dRFU/dT was maximum and the normalized dRFU/dT was greater than 0.3.

Software. Commercially available real-time cyclers use software designed to analyze reactions where fluorescence increases with PCR product accumulation. To analyze the results of real-time fluorescence quenching PCR reactions using this technology, novel software was developed. The software imports raw data from most real-time cyclers and performs cycle threshold and melt curve analyses to determine signal decrease during the amplification and signal change during the melt.

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