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DzyNA-PCR: Use of DNAzymes to Detect and Quantify Nucleic Acid Sequences in a Real-Time Fluorescent Format

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Background: **DzyNA-PCR is a general strategy for the detection and quantification of specific genetic sequences associated with disease or the presence of foreign agents. The method allows homogeneous gene amplification coupled with signal detection in a single closed vessel.**

Methods: **The strategy involves in vitro amplification of genetic sequences using a DzyNA primer that harbors the complementary (antisense) sequence of a 10-23 DNAzyme. During amplification, amplicons are produced that contain active (sense) copies of DNAzymes that cleave a reporter substrate included in the reaction mixture. The accumulation of amplicons during PCR can be monitored in real time by changes in fluorescence produced by separation of fluoro/quencher dye molecules incorporated into opposite sides of a DNAzyme cleavage site within the reporter substrate. The DNAzyme and reporter substrate sequences can be generic and hence can be adapted for use with primer sets targeting various genes or transcripts.**

Results: **Experiments using K-*ras* plasmid as template demonstrated that DzyNA-PCR allows quantification of DNA over at least six orders of magnitude ($r = 0.992$). Studies with human genomic DNA demonstrated the ability to resolve as little as twofold differences in the amount of starting template. DzyNA-PCR allowed the detection of 10 or fewer copies of the target. The clinical utility of the assay was demonstrated using DzyNA-PCR to analyze DNA that was isolated from human serum.**

Conclusion: **DzyNA-PCR is a simple, rapid, and sensitive technique for homogeneous amplification and quantification of nucleic acids in clinical specimens.**

In vitro evolution has been used to discover nucleic acids capable of catalyzing a broad range of reactions. The 10-23 DNAzyme is capable of cleaving nucleic acid substrates at specific RNA phosphodiester bonds under simulated physiological conditions *(1)*. This DNAzyme has a catalytic domain of 15 deoxynucleotides flanked by two substrate-recognition domains (arms). The DNAzyme interacts with the substrate through Watson-Crick pairing and cleaves between an unpaired purine and a paired pyrimidine. These molecules have potential as therapeutic agents through suppression of gene function by inactivation of target cellular RNA *(1–3)*. DNAzymes can also be exploited as molecular tools in genetic diagnostic assays.

This study describes the use of 10-23 DNAzymes to facilitate the detection of the products of in vitro amplification by PCR *(4, 5)*. The strategy, which is known as DzyNA-PCR, is illustrated in Fig. 1. PCR is performed using a DzyNA primer that contains a target-specific sequence and the complementary (antisense) sequence of a 10-23 DNAzyme. During PCR, amplicons are generated that contain both target sequences and active (sense) copies of DNAzymes. A DNA/RNA chimeric reporter substrate, containing fluorescence resonance energy transfer fluorophores incorporated on either side of a DNAzyme cleavage site, is included in the PCR mixture. Cleavage of this reporter substrate produces an increase in fluorescence that is indicative of successful amplification of the target gene or transcript. DzyNA-PCR is a generic and flexible strategy that provides an alternative to other homogeneous amplification and detection systems, including the TaqMan™ *(6)*, Molecular Beacon *(7)*, Sunrise™ primer *(8)*, and HybProbe assays *(9)*.

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Materials and Methods

PCR PRIMERS

The 5′ PCR primer 5K49 (5′-CCTGCTGAAAATGACTGAATATAAA-3′) is complementary to the human K-*ras* gene. The 3′ primer 3K45Dz2 (5′-**CCACTC***TCGTTGTAGCTAGCC***TATTAGCTGTATCGTCAAGCCACTC**TTGC-3′) is a DzyNA-PCR primer that contains (*a*) a 5′ region containing the catalytically inactive antisense sequence complementary to an active 10-23 DNAzyme (plain bold text indicates the complement of the arms that hybridize to the reporter substrate, and italic text indicates the complement of the 10-23 catalytic domain), and (*b*) a 3′ region that is complementary to the human K-*ras* gene (underlined). The 3′ primer 3K45 (5′-ATTAGCTGTATCGTCAAGCCACTC-3′) is a control primer that contains the same K-*ras*-specific sequence that is incorporated in 3K45Dz2; however, 3K45 is a standard PCR primer and does not contain the antisense sequence of a DNAzyme. Primers were synthesized by Pacific Oligos (Lismore NSW, Australia) or GeneWorks (Adelaide SA, Australia).

REPORTER SUBSTRATES

The DzyNA reporter substrate SubDz2 (5′-CCACTCguATTAGCTGTATCGTCAAGCCACTC-3′) is a chimeric oligonucleotide containing both RNA (lower case) and DNA bases. The substrate is designed such that the bond between the GU ribonucleotides is cleaved by active DNAzymes generated during DzyNA-PCR. Two versions of the substrate were synthesized, SubDz2 FAM/TAMRA and SubDz2 FAM/DABCYL, where the reporter 6-carboxyfluorescein (FAM)[1] was incorporated at the 5′ end, and the quencher 6-carboxytetramethylrhodamine (TAMRA) or 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL) was incorporated internally at nucleotide 10. A 3′-phosphate group was added to prevent extension by DNA polymerase during PCR. The substrates were synthesized by Oligos Etc., Inc. (Wilsonville, OR).

DNA TEMPLATES FOR PCR

The plasmid pCRKM contained the genomic sequence between nucleotides 84 and 289 of the human cellular c-Ki-*ra*s2 protooncogene, exon 1 (GenBank Locus HUMRASK02, Accession no. L00045) cloned into the vector pCR2.1 (Original TA cloning kit; Invitrogen). Plasmid was purified by column chromatography (Qiaprep Spin Plasmid kit; Qiagen) and used as a reference to establish the calibration curve used for quantification. The starting copy number was determined from measurements of the absorbance at 260 nm (A_{260}), and a range of calibrators from 10^7 to 10^0 copy number was generated from serial dilutions of plasmid DNA. Genomic DNA was extracted from the human leukemic cell line K562 and from human serum specimens according to the cationic polymer protocol *(10)*. The cell line K562 was obtained from the American Type Culture Collection (CCL 243). Serum was collected with informed consent from patients with pancreatic or colorectal cancer who were undergoing treatment at St Vincent's Hospital (Sydney, Australia).

AMPLIFICATION AND DETECTION

Thermal cycling and monitoring of fluorescence during PCR and data analysis were carried out using the ABI PRISM® 7700 Sequence Detection System (SDS; PE Biosystems), MicroAmp® optical 96-well reaction plates, and MicroAmp optical caps (PE Biosystems). PCR reactions contained 0.4 μmol/L 5K49, 0.06 μmol/L 3K45Dz2, 0.2 μmol/L SubDz2, 8 mmol/L $MgCl_2$, 100 mmol/L each dNTP (dATP, dCTP, dGTP, and dTTP), and 20 U of rRNasin (Promega Corporation) per 50-μL reaction. Reactions shown in Figs. 2 and 4 contained Gold buffer and 6 U of AmpliTaq Gold DNA polymerase (PE Biosystems). Reactions shown in Fig. 3 contained 10 mmol/L Tris-HCl, 75 mmol/L KCl, pH 8.3 (at 25 °C), as buffer and 3 U of AmpliTaq DNA polymerase (PE Biosystems) preincubated with TaqStart™ antibody (Clontech) in the ratio 1:10 according to manufacturer's instructions. Thermocycling conditions were as described in the figure legends. Template DNA samples analyzed by DzyNA-PCR were pCRKM plasmid DNA (10-fold serial dilutions from 10^7 to 10^0 copy number; Figs. 2 and 4), genomic DNA isolated from the cell line K562 (31–500 ng; Fig. 3), and genomic DNA isolated from the equivalent of 5 μL of human serum (Fig. 2B). All reactions were performed in duplicate unless otherwise indicated. Additional control reactions were performed in parallel and contained all reaction components with the following changes: no-template DzyNA control (NTC) mixtures lacked template DNA, and standard PCR control mixtures lacked the primer 3K45Dz2, which was replaced by the 3K45 control primer.

DATA ANALYSIS

Sequence detection software (PE Biosystems) was used to monitor the increase in FAM fluorescence at 530 nm following cleavage of substrate by amplicons harboring active DNAzymes. A cycle threshold value (Ct) was determined for each sample corresponding to the cycle when fluorescence exceeded a defined baseline signal (threshold ΔRn) within the log phase of product accumulation. A calibration curve was generated when the log of the copy number was plotted against the Ct value. Quantification of the amount of DNA in reactions containing unknown numbers of copies of the K-*ras* gene was estimated from the calibration curve. Baseline settings for analysis were in the cycle number 2–10 range. Analysis using sequence detection software was performed in the absence of correction for the passive reference 6-carboxy-X-rhodamine because this was not included in the DzyNA-PCR mixtures.

[1] Nonstandard abbreviations: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; DABCYL, 4-(4′-dimethylaminophenylazo)benzoic acid; SDS, sequence detection system; Ct, threshold cycle; and NTC, no-template control.

Results and Discussion

DzyNA-PCR strategy

This report describes the development and evaluation of a novel technology, DzyNA-PCR, to amplify and detect a region of exon 1 of the human K-*ras* gene in a homogeneous real-time format. PCR components included a DzyNA 3′ primer (3K45Dz2), which harbors the antisense sequence of a 10-23 DNAzyme, a 5′ primer (5K49), and a dual-labeled fluorogenic reporter substrate (SubDz2; Fig. 1). Successful amplification of target DNA, and concomitant cleavage of the reporter substrate by amplicons containing active DNAzymes, was indicated by an increase in fluorescent emission of the FAM reporter (Figs. 2–4). The fluorescent signal was monitored at each anneal/extend step, and quantification of template copy number was determined using an ABI PRISM 7700 SDS.

The 3′ primer 3K45 was used in standard PCR control reactions to amplify K-*ras* amplicons lacking the 10-23 catalytic core. No increase in fluorescent signal above background was observed in these control reactions. This control confirms that the increases observed during DzyNA-PCR are attributable to the presence of DNAzyme sequences within the amplicons and not to hydrolysis of the reporter substrate as a result of either high temperatures or the presence of contaminating RNases. Additional experiments using ^{32}P-labeled substrate confirmed that the mechanism of action of DNAzymes generated during DzyNA-PCR is genuine cleavage of the substrate. The presence of intact and cleaved ^{32}P-labeled substrate was visualized by phosphoro-imagery after separation of fragments by polyacrylamide electrophoresis (data not shown).

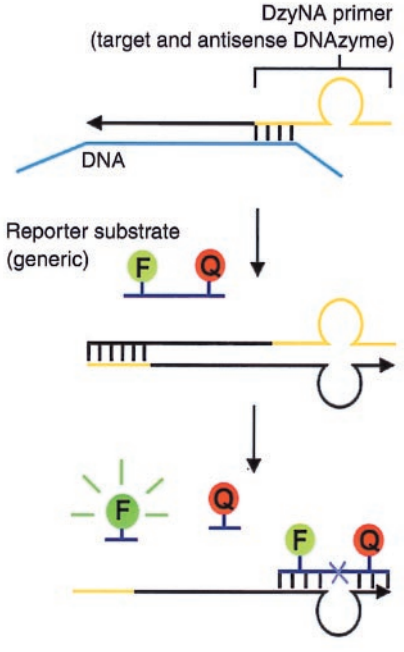

Fig. 1. DzyNA-PCR strategy for homogeneous amplification and detection of specific nucleic acid sequences.

The DzyNA primer contains a target-specific sequence and harbors the antisense sequence of a 10-23 DNAzyme. During amplification, amplicons are produced that contain active (sense) copies of DNAzymes that cleave a reporter substrate included in the reaction mixture. The accumulation of amplicons during PCR is monitored by an increase in reporter fluorescence produced by separation of fluoro/quencher dye molecules incorporated into opposite sides of a DNAzyme cleavage site within the reporter substrate.

Characteristics of DzyNA-PCR assay

The linear range, detection limit, specificity, reproducibility, and accuracy of quantification of the DzyNA-PCR assay were assessed. To determine the linear range and to develop a calibration curve for purposes of quantification, 10-fold serial dilutions of plasmid DNA (pCRKM) consisting of 10^7–10^0 target copy number were analyzed. As shown in Fig. 2, the dynamic linear range was at least six orders of magnitude, from 10^7 to 10^1 target copy number. The correlation coefficient of the calibration curve was consistently 0.99 or greater. The assay was specific for target DNA as shown by the high threshold cycle numbers (Ct values) of the NTCs, which were consistently at least 10 cycles greater than those observed in reactions containing 10 target molecules, as determined in consecutive assays. The reproducibility of Ct values obtained for

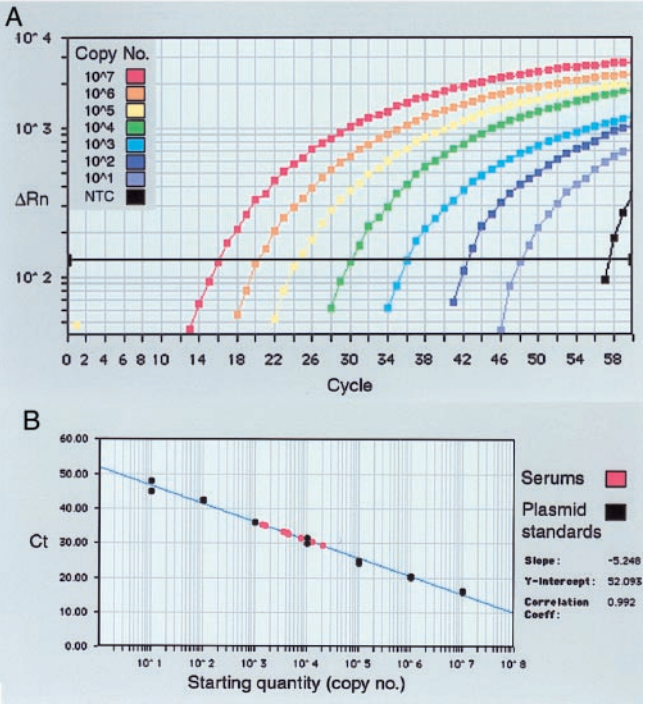

Fig. 2. Quantification of K-*ras* sequences using SubDz2 FAM/TAMRA reporter substrate.

(*A*), amplification plots of 10-fold serial dilutions of K-*ras* plasmid DNA containing 10^7–10^1 starting copy number and a NTC. Cycling conditions were 1 cycle of 95 °C for 15 min, 10 cycles of 65 °C for 1 min and 94 °C for 1 min, followed by 60 cycles of 40 °C for 1 min and 94 °C for 15 s. Fluorescence data were recorded during annealing at 40 °C; hence, cycle numbers as shown exclude the initial 10 cycles at 65 °C. Δ*Rn* represents the signal produced by reporter fluorescence minus the average baseline signal between cycles 5 and 8. Threshold ΔRn was set at 130. (*B*), calibration curve generated using SDS software where *Ct* (derived from *A*) is plotted against starting quantity (copy number; $r = 0.992$). Unknowns (*red*) are samples of DNA extracted from serum of patients with gastrointestinal malignancies.

a given number of copies of plasmid DNA was assessed within a single assay by testing replicate reactions containing 10^5 or 10^3 copy number. The CVs of the Ct values of these replicate measurements were 3.3% (n = 11), and 2.7% (n = 10), respectively.

Similar calibration curves were generated using 10-, 5-, and 2-fold serial dilutions of genomic DNA. DzyNA-PCR was shown to be capable of resolving twofold dilutions of genomic DNA across the range 31–500 ng per 50 μL (r = 0.980; Fig. 3). During PCR, the number of amplicons doubles with each cycle; hence determination of twofold differences in the amount of starting template corresponds to the theoretical limit of resolution for any PCR-based assay.

CLINICAL APPLICATION

DzyNA-PCR was used to estimate the concentrations of circulating K-*ras* DNA in the serum of patients with gastrointestinal malignancies (Fig. 2B). Serum samples (5 μL) were estimated to contain between 10^3 and 10^4 copies of a single-copy gene (K-*ras*), indicating that the concentrations of circulating genomic DNA in these patients were in the range of 0.8–4 mg/L serum. DzyNA-PCR therefore allowed quantification of circulating DNA using only small amounts of the clinical specimens. The ability to detect tumor markers, such as microsatellite instability, aberrant methylation, or mutated oncogenes, in serum or plasma may provide a new noninvasive tool for diagnosis, prognosis, and follow-up of cancer *(11–14)*. Highly sensitive techniques such as restriction endonuclease-mediated selective PCR *(10, 15)* and methylation-specific PCR *(16)*, which allow detection of activated alleles in a 1000-fold excess of wild-type alleles, are likely to be most compatible with noninvasive diagnostic approaches. DzyNA-PCR can be used to ascertain whether the amount of circulating DNA extracted is adequate to ensure that the sensitivity of subsequent analyses by these techniques is not compromised by the addition of insufficient DNA in reaction mixtures *(17)*. Furthermore, it is technically feasible to use restriction endonuclease-mediated selective PCR or methylation-specific PCR primers in conjunction with DzyNA primers for direct detection of genetic abnormalities in serum samples.

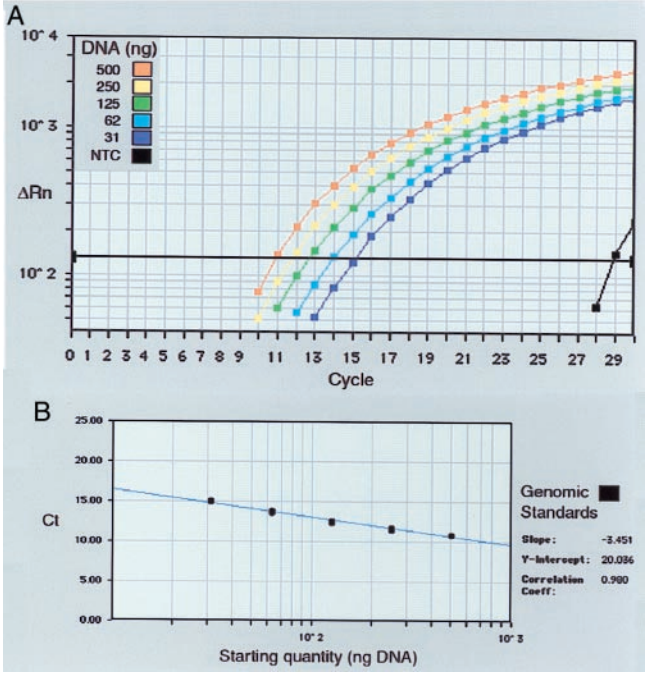

Fig. 3. Quantification of K-*ras* sequences using SubDz2 FAM/TAMRA reporter substrate and twofold dilutions of genomic DNA template.

(*A*), amplification plots of K-*ras* genomic DNA containing 31–500 ng as the starting quantity and a NTC. Cycling conditions were 1 cycle of 94 °C for 3 min, 20 cycles of 70 °C (−1.0 °C per cycle) for 1 min and 94 °C for 5 s, followed by 50 cycles of 40 °C for 1 min and 94 °C for 5 s. Fluorescence data were recorded during annealing at 40 °C; hence, cycle numbers as shown exclude the initial 20 touchdown cycles at 70 °C. Δ*Rn* represents the signal produced by reporter fluorescence minus the average baseline signal between cycles 2 and 8. Threshold ΔRn was set at 130. (*B*), calibration curve generated using SDS software where *Ct* (derived from *A*) is plotted against starting quantity (ng DNA; r = 0.980).

POTENTIAL FOR MULTIPLEX DzyNA-PCR

Future work in this laboratory will aim at developing multiple generic substrates, each of which could be labeled with a different fluorophore and each of which could be adapted to report on the presence of specific target amplicons. The simultaneous use of multiple substrates in a single reaction mixture would allow the development of multiplex DzyNA-PCR assays. In preliminary work, our initial substrate (SubDz2) was resynthesized with DABCYL replacing the TAMRA moiety. DABCYL previously has been exploited as a universal quencher on molecular beacons *(7)*. In a study by Vet et al. *(18)*, use of this nonfluorescent quencher allowed the resolution of four molecular beacons with different fluorophores in a single reaction mixture with analysis by the ABI PRISM 7700.

The substrate SubDz2 FAM/DABCYL was used in the DzyNA-PCR system targeting K-*ras* to resolve 10-fold dilutions from 10^7 to 10^0 copy number (r = 0.995; Fig. 4). In reactions containing template diluted to contain only a single target molecule, the presence of K-*ras* was detected in some but not all reactions (Fig. 4B). This is likely to reflect sporadic sampling of single or small numbers of target molecules. SubDz2 FAM/DABCYL was more compatible with stringent thermocycling profiles containing annealing/extension temperatures of 50 °C than was the SubDz2 FAM/TAMRA substrate. This may be the result of enhanced stabilization of the secondary structure of the hybridizing arms at the higher temperature when the DABCYL moiety replaces TAMRA.

FACTORS INFLUENCING DzyNA-PCR ASSAY EFFICIENCY

Before this study, investigations into the ability of the 10-23 DNAzyme to cleave substrates had largely been confined to physiological conditions. The conditions required for PCR vary greatly from the physiological with respect to a number of conditions such as temperature,

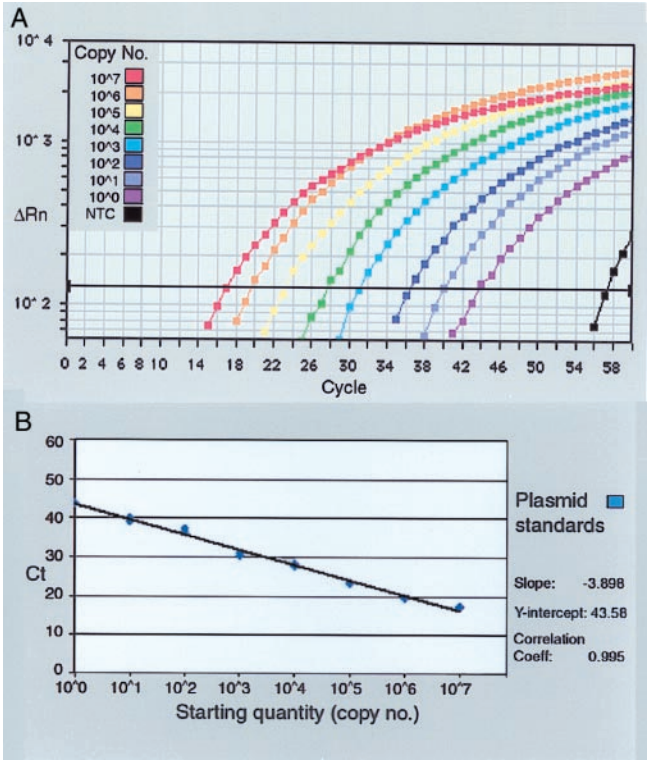

Fig. 4. Quantification of K-*ras* sequences using SubDz2 FAM/DABCYL reporter substrate.

(*A*), amplification plots of 10-fold serial dilutions of K-*ras* plasmid DNA containing 10^7–10^0 starting copy number and a NTC. Cycling conditions were 1 cycle of 95 °C for 15 min, 10 cycles of 65 °C for 1 min and 94 °C for 1 min, followed by 60 cycles of 50 °C for 1 min and 94 °C for 15 s. Fluorescence data were recorded during annealing at 50 °C; hence, cycle numbers as shown exclude the initial 10 cycles at 65 °C. Δ*Rn* represents the signal produced by reporter fluorescence minus the average baseline signal between cycles 2 and 10. Threshold ΔRn was set at 130. (*B*), calibration curve where *Ct* (derived from *A*) is plotted against starting quantity (copy number; $r = 0.995$). Ct values were duplicates, with the exception of 10^0 copy number, and were exported to Microsoft Excel for analysis because the SDS software requires that probes be labeled with a FAM reporter and a TAMRA quencher to calculate the calibration curve automatically.

ionic strength, and buffer composition. In DzyNA-PCR, reaction conditions must be compatible with both amplification by PCR and cleavage by the 10-23 DNAzyme. A range of suitable conditions was identified in a series of experiments where the DNAzyme-mediated cleavage of ^{32}P-labeled substrates was quantified by phosphoro-imagery (H. Impey et al., manuscript in preparation). In DzyNA-PCR, the primer concentrations were shown to affect the efficiency and were adjusted to favor production of the strand containing the active DNAzymes.

The efficiency of cleavage by 10-23 DNAzymes can be influenced by the length of their hybridizing arms *(1)*. Amplicons produced by the K-*ras* DzyNA-PCR system described above harbored DNAzymes that hybridized to SubDz2 via one short 6-base arm and one long 25-base arm. In initial experiments using SubDz2 FAM/TAMRA, annealing temperatures of 40 °C were used to promote efficient hybridization of the short arm of the DNAzyme in an effort to ensure efficient cleavage. Subsequent experiments using SubDz2 FAM/DABCYL demonstrated that SubDz2 could be efficiently cleaved during PCR incorporating higher annealing temperature steps (50 °C) and that the quencher moiety used to label the substrate could influence the efficiency. Additional studies in this laboratory have shown that DzyNA-PCR systems that generate DNAzymes with longer hybridizing arms facilitate highly efficient cleavage at temperatures ranging between 50 and 60 °C (H. Impey et al., manuscript in preparation). The inherent catalytic activity of the 10-23 DNAzyme is therefore retained at high temperatures such as those typically used during the annealing phase of PCR. Release of the substrate from the DNAzyme after cleavage is ensured by the high temperature used for the denaturation step in PCR.

FEASIBILITY FOR CLINICAL DIAGNOSTICS

Homogeneous sealed-tube formats have several advantages over protocols where amplicons are analyzed separately after amplification. Closed system methods are faster and simpler because they require fewer manipulations. Furthermore, such systems eliminate the potential for false positives associated with contamination with PCR products from other reactions. In DzyNA-PCR, the reporter substrate and the corresponding primer sequence encoding the DNAzyme can be generic. As such, new DzyNA-PCR assays could be developed more rapidly than assays such as TaqMan *(6)*, Molecular Beacons *(7)*, and HybProbes *(9)*. These assays depend on internal hybridization of reporter probes that must be developed for each target nucleic acid.

Theoretically, the sequence encoding a generic DNAzyme could be attached to any primer targeting a gene or transcript. In practice, primers are selected for DzyNA assays on the basis of lack of propensity for formation of catalytically active primer-dimers. Not all primer-dimers are expected to harbor catalytically active DNAzymes because this requires the primers to anneal in such a way as to copy the entire catalytic domain and a substantial portion of the hybridizing arm sequence. This provides an advantage over the Sunrise *(8)* strategy where all primer-dimers produce increases in reporter fluorescence. When DzyNA-PCR is monitored in real time, increases in the fluorescence attributable to primer-dimers can be clearly distinguished from the fluorescence associated with amplification of target nucleic acids. The Ct values produced by active primer-dimer formation in reactions lacking template DNA are consistently at least 10 cycles greater than those observed in reactions containing 10 target nucleic acid molecules.

DzyNA PRIMER SYSTEMS: COMPATIBILITY WITH ALTERNATIVE AMPLIFICATION STRATEGIES

The general strategy outlined here is potentially very flexible. In addition to PCR, several other strategies for in vitro amplification of nucleic acid sequences have been described. These include strand displacement amplifica-

tion *(19)*, which produces DNA products, and transcription-mediated amplification *(20)*, which produces RNA products. Theoretically, the catalytic nucleic acid molecule encoded by a DzyNA primer could be either a DNAzyme if PCR or strand displacement amplification were used, or a ribozyme (catalytic RNA enzyme) if transcription-mediated amplification was used to mediate nucleic acid amplification. Furthermore, in vitro evolution technology has facilitated the discovery of DNAzymes and ribozymes capable of catalyzing a broad range of reactions, including cleavage *(1, 21–23)* and ligation of nucleic acids *(24)*, porphyrin metallation *(25)*, and the formation of carbon-carbon *(26)*, ester *(27)*, or amide bonds *(28)*. Therefore, it may be possible to develop systems for detection of in vitro amplification products where the reporter substrate is a molecule other than a nucleic acid and/or the readout of the assay is dependent on a modification other than cleavage of the substrate.

The discovery of the 10-23 DNAzyme has provided a new tool for use in genetic diagnostics. This study has demonstrated the use of DNAzymes to facilitate homogeneous nucleic acid amplification, detection, and quantification in a real-time fluorescent format. Methods of in vitro nucleic acid amplification have widespread applications in genetics, disease diagnosis, and forensics. The development of rapid, homogeneous systems for amplification and detection are required to facilitate the transfer of molecular diagnostics from the laboratory to the clinic.

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