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A novel photoinduced electron transfer (PET) primer technique for rapid real-time PCR detection of *Cryptosporidium* spp.

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ABSTRACT

We report the development of a fluorescently labeled oligonucleotide primer that can be used to monitor real-time PCR. The primer has two parts, the 3′-end of the primer is complimentary to the target and a universal 17-mer stem loop at the 5′-end forms a hairpin structure. A fluorescent dye is attached to 5′-end of either the forward or reverse primer. The presence of guanosine residues at the first and second position of the 3′ dangling end effectively quenches the fluorescence due to the photo electron transfer (PET) mechanism. During the synthesis of nucleic acid, the hairpin structure is linearized and the fluorescence of the incorporated primer increases several-fold due to release of the fluorescently labeled tail and the absence of guanosine quenching. As amplicons are synthesized during nucleic acid amplification, the fluorescence increase in the reaction mixture can be measured with commercially available real-time PCR instruments. In addition, a melting procedure can be performed to denature the double-stranded amplicons, thereby generating fluorescence peaks that can differentiate primer dimers and other non-specific amplicons if formed during the reaction. We demonstrated the application of PET-PCR for the rapid detection and quantification of *Cryptosporidium parvum* DNA. Comparison with a previously published TaqMan® assay demonstrated that the two real-time PCR assays exhibited similar sensitivity for a dynamic range of detection of 6000–0.6 oocysts per reaction. PET PCR primers are simple to design and less-expensive than dual-labeled probe PCR methods, and should be of interest for use by laboratories operating in resource-limited environments.

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1. Introduction

The real-time polymerase chain reaction (PCR) has been extensively used as a diagnostic tool in clinical and environmental laboratories for detection and quantization of biological targets in a closed tube format. The simplest method of real-time PCR is the use of DNA intercalating dyes such as SYBR Green I dye [1,2] and LC Green [3] that enable real-time visualization of double-stranded DNA as it is produced during PCR.

The use of real-time PCR has been widely accepted as a standard practice in many laboratories for identifying target nucleic acids in a quantitative and specific manner. The real-time PCR is also user-friendly since analysis is completed without opening reaction tubes, thus minimizing potential post-PCR contamination. The detection of multiple targets in a single reaction tube is often desired to save sample, time and cost of analysis. The use of intercalating dyes for detecting multiple targets often fails to generate consistent melting profiles for multiple pathogens due to preferential binding and some intercalating dyes have also been reported to inhibit PCR [4].

The specificity of real-time PCR can be increased by using a labeled sequence-specific probe. Several of such methods are currently available for performing real-time PCR, such as TaqMan® probes [5,6]; molecular beacons [7]; self-probing amplicons (scorpions) [8]; Amplisensor [9]; Amplifluor [10]; displacement hybridization probes [11]; fluorescence resonance energy transfer (FRET)-TaqMan probes [12]; DzyNA-PCR [13]; fluorescent restriction enzyme detection [14]; and adjacent hybridization probes [15]. One of the major limitations of currently available real-time PCR technologies is cost. Probe-based real-time PCR requires the addition of fluorophore and quencher dyes to oligonucleotide probes. Because of the need for dual labeling, such probes are relatively expensive. An alternative to the dual-labeling technique is the use of the fluorescence quenching properties of guanosine (enabled by guanosine's electron donating properties) [16,17]. The electron donating mechanism has been referred to as photoinduced electron transfer (PET) and has been employed in real-time PCR applications [18]. Applications of PET primers have been reviewed [19]. Single-labeled fluorescein probes that are quenched

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by target hybridization have been reported by Crockett and Wittwer et al. [20] for melt curve analysis. Several real-time PCR chemistries based on guanosine quenching have also been reported in the literature [18,20–24].

Fluorescein is the most commonly used fluorophore that can be quenched by guanosine bases, but the extent of quenching is affected by the location of guanosine in relation to the fluorophore. For example, studies have shown that the quenching effect for an assay can be influenced by the number of guanosines and the guanosine position relative to fluorophore in the same strand [25] or in hybridizing strand [21,26] or due to overhang region [17,21,27].

We report here the development of a fluorescently labeled oligonucleotide primer that can be used to monitor real-time PCR. The fluorescent primer in this study is referred to as photoinduced electron transfer primer and named as "PET" primer for easy reference. The PET assay employs a single pair of primers, with one of the primers labeled with a single fluorophore dye at its 5′-end and quenched by the presence of guanosines present in the overhang position of the primer. This method of labeling does not require HPLC purification (if FAM or HEX used), is commonly available, and reduces reagent costs since no quencher dye is required. The assay can be performed to monitor real-time fluorescence during amplification and enables use of melt curve analysis alone to monitor the reaction.

2. Methods

2.1. Principle of the assay

PET primers are comprised of two regions: (1) a target-specific primer region at the 3′ end and, (2) a universal stem-loop sequence at the 5′-end. For this study, the stem-loop sequence was comprised of 17 bases [TAMRA-5 AGGCGCataGCGCCTgg (i.e., the fluorophore TAMRA was attached at the 5′-end adenine)]. This stem-loop sequence incorporated a loop of six bases (underlined), a 6 base-pair stem (CGCGGA/GCGCCT) complimentary structure, a 3-nucleotide loop (ATA) and two deoxyguanosine bases located in the opposite overhang region (Fig. 1A). AGGCGC, ATA and GCGCCT together form the stem-loop-like structure. In the absence of amplification, the loop-tail bases of the PET primer sequences remain in a closed form and are quenched by two overhang GG based and two complementary GG residues in the hairpin formation. TAMRA fluorescence is effectively quenched due to close proximity of the four G based to the fluorophore. Upon the participation of the PET primer in nucleic acid amplification, the stem-loop structure opens up and fluorescence increases due to a conformational change when extension of the complimentary strand takes place (Fig. 1B). The fluorescence increase is due to the de-quenching effect that occurs when guanosine bases located in overhang positions are de-coupled from the stem-loop structure and are incorporated into the double-stranded product.

Both forward and reverse primers are designed with a T_m value of approximately 60 °C. The universal stem loop structure is added to either the forward or reverse primer having the maximum ΔG (kcal/mol) at the 3′ end. A higher ΔG value means the oligonucleotide is more thermodynamically stable. The seven bases at the 3′ end of the forward and reverse primers are used to compare maximum ΔG values between the primers, based on the Nearest-Neighbor parameter values using the software, Oligo Analyzer 1.1.2 (Freeware, Teemu Kuulasmaa, Finland). The universal stem-loop sequence is added to the primer that has the higher ΔG value. In the present study, the reverse primer was selected (ATTCCCCGTTACCCGTCA) for incorporation of the stem-loop sequence because this primer had a higher ΔG value (−13.1 kCal/mol) than the forward primer (−10.0 kCal/mol).

2.2. Primers for PET PCR amplification

We selected *Cryptosporidium parvum* as a model to demonstrate the application of the present PET primer technique for real-time PCR. The HPLC purified fluorescent labeled primer was synthesized at CDC's Biotechnology Core Facility. TAMRA-dT was coupled to the oligo at the 5′-end of the reverse primer during synthesis to produce the end labeled primer of sequence 5′(TAMRA)-AGGCGCATAGCGCCTGG. The tail does not show any homology to *Cryptosporidium* spp. sequences. The modified reverse primer consisted of 35 bases including a labeled hairpin oligonucleotide of 17 bases 5′(TAMRA)-AGGCGCATAGCGCCTGGATTCCCCGTTACCCGTCA (CryJVR; Position #119-102). The unlabeled forward primer consisted of a base sequence of GGTGACTCATAATAACTTTACGGAT (CryJVF; Position #1-25). These primer sequences correspond to *C. parvum* 18S ssrRNA (GenBank Accession #JQ182992). The CryJVR/CryJVF primers amplify a 119-bp sequence specific to *Cryptosporidium* spp.

2.3. PET PCR assay conditions

PET PCR was carried out using the iCycler iQ4 (Bio-Rad, CA, USA) platform for a total of 45 cycles. The reaction mixture contained primers at concentrations of 250 nM of each forward and reverse primer, 2 μl of DNA, 10 μl of 2× QuantiTect Probe PCR kit Master Mix (Qiagen, Valencia, CA), and nuclease-free water to a final volume of 20 μl. The amplification reaction consisted of a hot start step at 95 °C for 15 min to activate the HotStart Taq DNA polymerase. This was followed by 45 cycles of amplification including denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 50 s. Fluorescence signals were collected at the end of the annealing step. Melting curve analysis was performed after amplification by cooling to 48 °C for 10 s, followed by a temperature increase to 97.5 °C, while continuously collecting the fluorescence signal data at 0.5 °C increments. The melt curve analysis generated a characteristic single peak at melting temperature (T_m) and used to confirm amplification of the target *Cryptosporidium* sequence using the iCycler® data analysis software.

2.4. TaqMan PCR assay conditions

The TaqMan assay reported by Jothikumar et al. was used [28]for comparison to the PET PCR assay. The TaqMan probe was labeled with FAM (6- Carboxy-fluorescein) at the 5′-end and with black hole quencher at the 3′ end (CDC's Biotechnology Core Facility). Amplifications were carried out using the iCycler (Bio-Rad, California, USA) for a total of 45 cycles. For TaqMan PCR, the 20-μl reaction contained 10 μl of 2× QuantiTect Probe PCR kit Master Mix (Qiagen, Valencia, CA), 2 μl of DNA, and primers and probe at concentrations of 250 and 100 nM respectively. Prior to amplification, denaturation was carried out at 95 °C for 15 min, followed by 45 PCR cycles at 95 °C (10 s) and annealing/extension at 60 °C for 50 s. Fluorescence signals were collected at the end of annealing step.

2.5. Real-time quantification

A *C. parvum* stock containing 5×10^8 oocysts (Iowa isolate) was obtained from Waterborne, Inc. (New Orleans, LA, USA). DNA was extracted from the stock using bead beating and lysis buffer protocol reported by Hill et al. [29]. Replicate standard curves were generated using 10^3 to 10^0 oocysts. For generation of standard curves, the crossing threshold (C_T) values were plotted proportionally to the logarithm of the input copy numbers. Appropriate negative controls were included in each run. To assess the log-linear relationship of the assays, the linear regression and regression

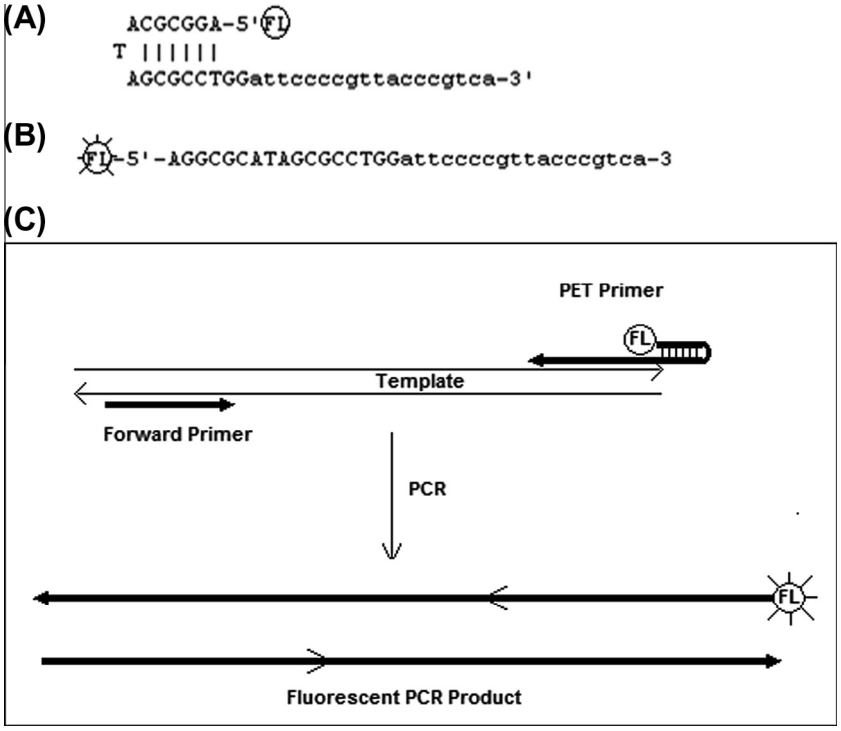

Fig. 1. Schematic representation of PCR amplification using PET primer. (A) 5′-End fluorophore (FL) labeled PET primer (upper case letters) remains non-fluorescent in stem-loop format and the signal of the fluorophore is quenched by two consecutive G nucleotides located at overhang and complementary regions due to the close proximity of the fluorophore and guanosines. The target-specific sequence region of the reverse primer is shown in small case letters. (B) Stem-loop becomes linear during the amplification and increases the detectable signal. (C) During PCR amplification the stem-loop structure is disrupted and guanosine is displaced during the extension of the forward primer, resulting in formation of double-stranded product that is fluorescent.

coefficients (R^2) were calculated. PCR amplification efficiency (E) for both PET primer and TaqMan® probe assays were calculated according to the equation $E = 10^{(-1/\text{slope})}$-1. However, the oocysts numbers do not reflect the number of DNA molecules for 18S, since each oocyst contains 20 copies of 18S DNA molecules.

3. Results

3.1. Development of PET PCR assay

During nucleic acid amplification, the target-specific primer region is extended into the hairpin structure of the PET primer, eliminating the quenching mechanism. Fig. 1A and B shows a PET primer molecule when not hybridized to its complementary sequence (e.g., stem–loop structure present), and when hybridized to its complementary sequence (e.g., stem–loop structure absent), respectively. The fluorescence of the incorporated primer increases several-fold due to the unfolding of the 5′ stem–loop structure which moves the 5′ fluorophore away from the quenching properties of the guanosine bases. As more amplicons are produced during nucleic acid amplification, the overall fluorescence of the reaction mixture increases. The cyclic increase in fluorescence signal is the basis of the technique for detecting the presence of a target nucleic acid sequence in the reaction. The newly linearized template exhibits a several fold increase in florescence emission by the 5′ fluorophore (Fig 1C).

3.2. The sensitivity of the PET PCR assay

As shown in Fig. 2A, nucleic acid amplification with the PET PCR assay demonstrated a dynamic range of detection from 6000 to 0.6 oocysts per PCR reaction. As shown in Fig. 2B, the logarithmic plot of this data presents the relationship between the concentration of DNA and C_T values. In addition, a dissociation analysis of the PCR product can be performed by denaturation of double-stranded amplicons by a melting procedure, thereby obtaining fluorescence peaks specific for a product generated in the nucleic acid amplicons (Fig. 2C). For the *Cryptosporidium* spp. PET PCR assay, a single melt peak (T_m = 80.5 °C) for the specific product indicated the absence of non-specific amplification in the PCR products. The PET PCR assay was able to detect 0.6 *C. parvum* oocysts/reaction (Fig. 2A and B). The PET-PCR *Cryptosporidium* spp. assay was also performed with other fluorophores (FAM and HEX) and performed similarly as when TAMRA was used (data not shown).

3.3. Sensitivity comparisons of PET PCR and TaqMan assays

The TaqMan assay was also able to detect 0.6 *C.* parvum oocysts/reaction (Fig. 3A and B). Thus, the PET PCR and TaqMan assays achieved the same detection limit level. A seed level of 0.06 oocysts was not detected by either method. The reaction efficiency of the PET and TaqMan assays were 93.1% (slope −3.5) and 91.6% (slope −3.54), respectively.

4. Discussion

The present study describes a novel and cost effective rapid real-time PCR technique to detect target nucleic acid. This PET PCR technique requires only one of the primers to be fluorescently labeled (at the 5′-end). PET PCR primers incorporate a universal stem-loop structure that utilizes intentional locations of guanosine bases to yield low background signal in the absence of amplification, and high fluorescence signals when target DNA sequences are amplified during PCR. Several studies have reported the use of only one fluorophore for use in detecting the presence of a particular nucleic acid [17,18,20,30]. These studies have reported that

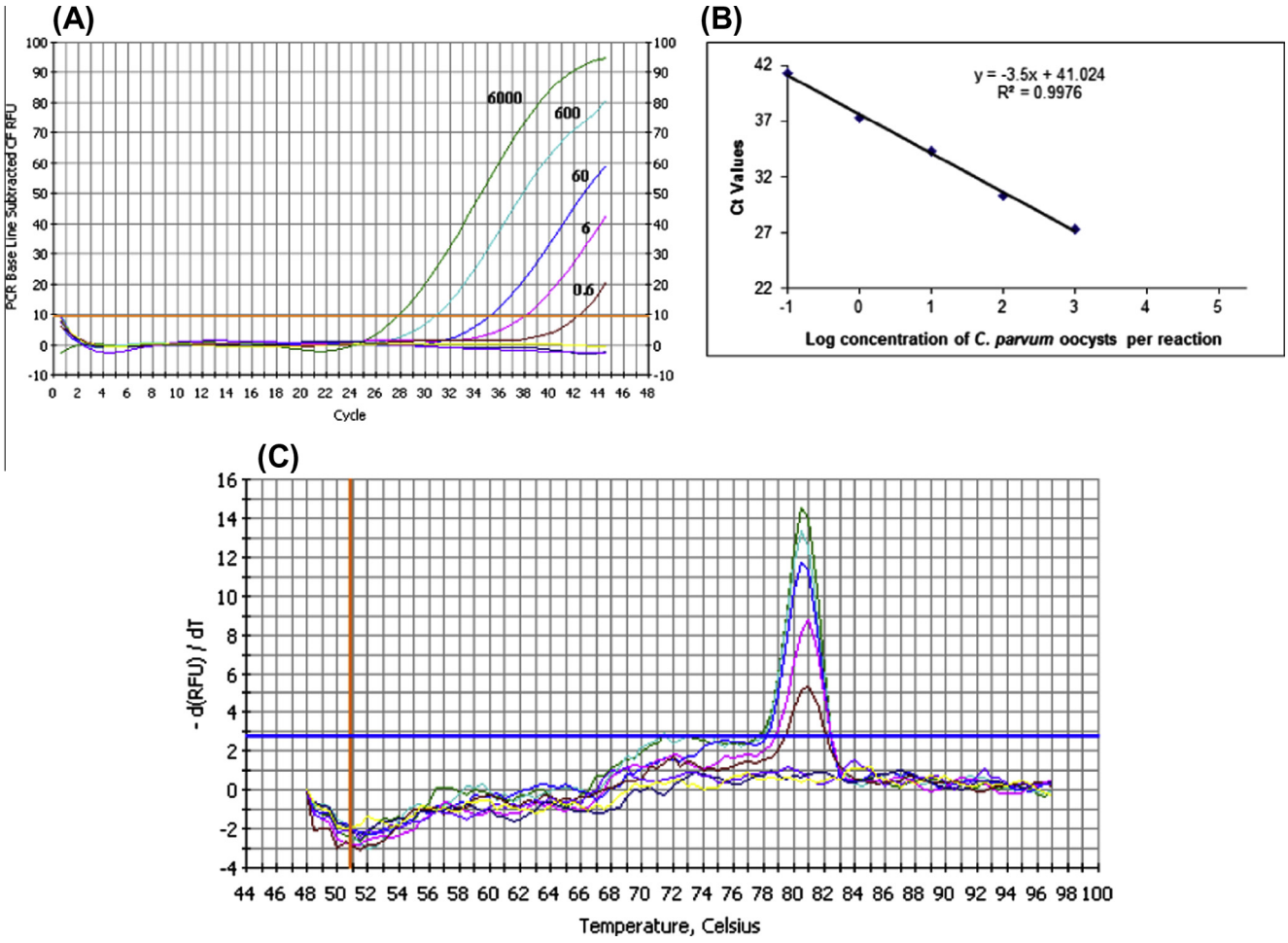

Fig. 2. Sensitivity of PET PCR assay. (A) Demonstrates the PET PCR assay dynamic range of detection from 6000 to 0.6 oocysts per PCR reaction. (B) Logarithmic standard curve plot for the *Cryptosporidium* spp. PET PCR assay depicting the relationship between the concentration of DNA in each reaction versus associated C_T values. (C) Melting curve analysis of the 18S ssrRNA target at the different concentrations shown in 2A and B, demonstrating consistent amplicon T_m.

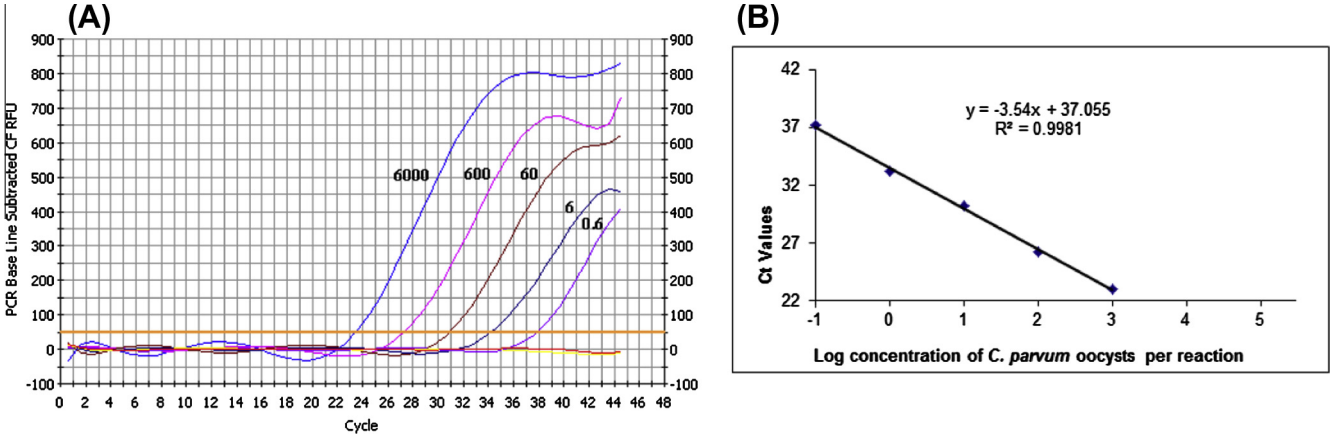

Fig. 3. Sensitivity of TaqMan assay. (A) Demonstrates the sensitivity of the PET primer is same as that of TaqMan assay along with logarithmic plot. TaqMan assay demonstrates the quantitative aspect of nucleic acid amplification for a dynamic range of detection from 6000 to 0.6 oocysts per PCR reaction. (B) Logarithmic plot of the above (TaqMan) data presents the relationship between the concentration of DNA and threshold cycle (C_T) values.

the fluorescence signal is either enhanced or quenched in the presence of the target nucleic acid sequence, depending on the particular design of the probe [31]. In most cases, the labeled primer specifically hybridizes to the target nucleic acid sequence. Similarly, Tam-Chang [32] describes a multi-probe universal reporter system containing a signal that is enhanced only after sequence-specific hybridization of one of the probes. Guo and Milewicz [33] reported the use of universal fluorescent tag primers labeled on the 5′-end that is not sequence specific. In this method, the labeled fluorescent tag universal primer, in combination with

two sequence-specific primers, is used to amplify a target nucleic acid sequence. Yamane [34] discusses a MagniProbe that has an internal fluorophore and an internal intercalator. The fluorescence is quenched by the intercalator dye in the absence of a target sequence and upon hybridization with the target sequence; the probe emits fluorescence due to the interference in quenching by intercalation. Nazarenko et al. [21] describe LUX primers with a single fluorophore near the 3′ end (but no quencher), and addition of 5–7 base pairs to the 5′-end of the sequence-specific probe, wherein the signal from the fluorophore is increased in the presence of the target sequence. Internal labeling of oligonucleotides (such as required for LUX primers) increases the cost of fluorescent primer production (versus end-labeling) and also requires specific software to design such primers. Phosphoramidites with FAM or HEX are widely available for labeling at the 5′-end, thus making production of PET primers simple and inexpensive.

In the present study, the PET PCR technique was used to create fluorescent primers for nucleic acid amplification of an 18S ssrRNA gene sequence specific to *Cryptosporidium* spp. The results (detection limit, PCR efficiency) were similar to a corresponding TaqMan assay targeting the same gene sequence. This result demonstrating similar sensitivity between a PET PCR assay and a corresponding TaqMan assay was also demonstrated by Naomi et al. for detection of malaria parasites [35]. Initially, we tested a large number of single-labeled oligonucleotides to develop a universal fluorescent tail sequence based on the combination of number of G's, melting curve, and high fluorescence yield. We determined that a 17-bp 5′-end fluorescent labeled sequence is optimal for PET PCR to enable detection sensitivity similar to the TaqMan technique. The identified 17-bp hairpin structure of the fluorescently labeled primer serves as a universal adapter and can be incorporated into existing PCR primer sequences for application in real-time PCR. The increase in fluorescence can be measured and observed using commercially available nucleic acid amplification systems. In addition, melt-curve analysis can be performed to confirm the production of target-specific amplicon.

There are several advantages of using PET primers for real-time PCR, including cost, sensitivity, speed, multiplexing, and real time monitoring of the amplicon in a closed-tube format. The cost of synthesis of a 5′-labeled fluorescent PET primer is relatively low because only a single fluorophore is needed and PET primers do not require HPLC purification if FAM or HEX is employed. This new technique represents a cost-effective alternative for real-time PCR and should enable sensitive detection of pathogens and other biological targets in clinical and environmental samples.

Competing interests

The authors are inventors on a pending US patent application PCT/US2008/084347 and international application covering real-time PET PCR.

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References

[1] K.M. Ririe, R.P. Rasmussen, C.T. Wittwer, Product differentiation by analysis of DNA melting curves during the polymerase chain reaction, Anal. Biochem. 245 (1997) 154–160.
[2] C.T. Wittwer, K.M. Ririe, R.V. Andrew, D.A. David, R.A. Gundry, U.J. Balis, The lightcycler: a microvolume multisample fluorimeter with rapid temperature control, Biotechniques 22 (1997) 176–181.
[3] C.T. Wittwer, G.H. Reed, C.N. Gundry, J.G. Vandersteen, R.J. Pryor, High-resolution genotyping by amplicon melting analysis using LCGreen, Clin. Chem. 49 (2003) 853–860.
[4] H. Gudnason, M. Dufva, D.D. Bang, A. Wolff, Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature, Nucleic Acids Res. 35 (2007) e127.
[5] L.G. Lee, C.R. Connell, W. Bloch, Allelic discrimination by nick-translation PCR with fluorogenic probes, Nucleic Acids Res. 21 (1993) 3761–3766.
[6] P.M. Holland, R.D. Abramson, R. Watson, D.H. Gelfand, Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of Thermus aquaticus DNA polymerase, Proc. Natl. Acad. Sci. USA 88 (1991) 7276–7280.
[7] S. Tyagi, F.R. Kramer, Molecular beacons: probes that fluoresce upon hybridization, Nat. Biotechnol. 14 (1996) 303–308.
[8] D. Whitcombe, J. Theaker, S.P. Guy, T. Brown, S. Little, Detection of PCR products using self-probing amplicons and fluorescence, Nat. Biotechnol. 17 (1999) 804–807.
[9] S. Chen, R. Xu, A. Yee, K.Y. Wu, C.N. Wang, S. Read, et al., An automated fluorescent PCR method for detection of shiga toxin-producing *Escherichia coli* in foods, Appl. Environ. Microbiol. 64 (1998) 4210–4216.
[10] I.A. Nazarenko, S.K. Bhatnagar, R.J. Hohman, A closed tube format for amplification and detection of DNA based on energy transfer, Nucleic Acids Res. 25 (1997) 2516–2521.
[11] Q. Li, G. Luan, Q. Guo, J. Liang, A new class of homogeneous nucleic acid probes based on specific displacement hybridization, Nucleic Acids Res. 30 (2002) E5.
[12] P. Jothikumar, V. Hill, J. Narayanan, Design of FRET-TaqMan probes for multiplex real-time PCR using an internal positive control, Biotechniques 46 (2009) 519–524.
[13] A.V. Todd, C.J. Fuery, H.L. Impey, T.L. Applegate, M.A. Haughton, DzyNA-PCR: use of DNAzymes to detect and quantify nucleic acid sequences in a real-time fluorescent format, Clin. Chem. 46 (2000) 625–630.
[14] M.J. Cairns, R. Turner, L.Q. Sun, Homogeneous real-time detection and quantification of nucleic acid amplification using restriction enzyme digestion, Biochem. Biophys. Res. Commun. 318 (2004) 684–690.
[15] C.T. Wittwer, M.G. Herrmann, A.A. Moss, R.P. Rasmussen, Continuous fluorescence monitoring of rapid cycle DNA amplification, Biotechniques 22 (130–1) (1997) 4–8.
[16] C.A.M. Seidel, A. Schulz, M.H.M. Sauer, Nucleobase specific quenching of fluorescent dyes 1 Nucleobase one-electron redox potentials and their correlation with static and dynamic quenching efficiencies, J. Phys. Chem.-US 100 (1996) 5541–5553.
[17] M. Torimura, S. Kurata, K. Yamada, T. Yokomaku, Y. Kamagata, T. Kanagawa, et al., Fluorescence-quenching phenomenon by photoinduced electron transfer between a fluorescent dye and a nucleotide base, Anal. Sci. 17 (2001) 155–160.
[18] S. Kurata, T. Kanagawa, K. Yamada, M. Torimura, T. Yokomaku, Y. Kamagata, et al., Fluorescent quenching based quantitative detection of specific DNA/RNA using a BODIPY (R) FL labeled probe or primer, Nucleic Acids Res. 29 (2001).
[19] J.P. Knemeyer, N. Marme, Recent patents on self-quenching DNA probes, Recent Pat. DNA Gene Seq. 1 (2007) 145–147.
[20] C.T. Wittwer, A.O. Crockett, Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides, Anal. Biochem. 290 (2001) 89–97.
[21] I. Nazarenko, R. Pires, B. Lowe, M. Obaidy, A. Rashtchian, Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes, Nucleic Acids Res. 30 (2002) 2089–2095.
[22] J.P. Knemeyer, M. Sauer, N. Marme, Probes for detection of specific DNA sequences at the single-molecule level, Anal. Chem. 72 (2000) 3717–3724.
[23] T. Maruyama, T. Shinohara, T. Hosogi, H. Ichinose, N. Kamiya, M. Goto, Masking oligonucleotides improve sensitivity of mutation detection based on guanine quenching, Anal. Biochem. 354 (2006) 8–14.
[24] C.P. Vaughn, K.S.J. Elenitoba-Johnson, Intrinsic deoxyguanosine quenching of fluorescein-labeled hybridization probes: a simple method for real-time PCR detection and genotyping, Lab Invest. 81 (2001) 1575–1577.
[25] H. Sigmund, T. Maier, W. Pfleiderer, A new type of fluorescence labeling of nucleosides, nucleotides and oligonucleotides, Nucleos. Nucleot. 16 (1997) 685–696.
[26] S.P. Lee, D. Porter, J.G. Chirikjian, J.R. Knutson, M.K. Han, Fluorometric assay for DNA cleavage reactions characterized with bamhi restriction-endonuclease, Anal. Biochem. 220 (1994) 377–383.
[27] J.E. Noble, L. Wang, K.D. Cole, A.K. Gaigalas, The effect of overhanging nucleotides on fluorescence properties of hybridising oligonucleotides labelled with Alexa-488 and FAM fluorophores, Biophys. Chem. 113 (2005) 255–263.
[28] N. Jothikumar, A.J. da Silva, I. Moura, Y. Qvarnstrom, V.R. Hill, Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays, J. Med. Microbiol. 57 (2008) 1099–1105.
[29] V.R. Hill, A.M. Kahler, N. Jothikumar, T.B. Johnson, D. Hahn, T.L. Cromeans, Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter tap water samples, Appl. Environ. Microbiol. 73 (2007) 4218–4225.

[30] A.O. Crockett, C.T. Wittwer, Fluorescein-labeled oligonucleotides for real-time pcr: using the inherent quenching of deoxyguanosine nucleotides, Anal. Biochem. 290 (2001) 89–97.
[31] S.A. Marras, F.R. Kramer, S. Tyagi, Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes, Nucleic Acids Res. 30 (2002) e122.
[32] S.W. Tam-Chang, T.D. Carson, L. Huang, N.G. Publicover, K.W. Hunter Jr., Stem-loop probe with universal reporter for sensing unlabeled nucleic acids, Anal. Biochem. 366 (2007) 126–130.
[33] D.C. Guo, D.M. Milewicz, Methodology for using a universal primer to label amplified DNA segments for molecular analysis, Biotechnol. Lett. 25 (2003) 2079–2083.
[34] A. Yamane, MagiProbe: a novel fluorescence quenching-based oligonucleotide probe carrying a fluorophore and an intercalator, Nucleic Acids Res. 30 (2002) e97.
[35] N.W. Lucchi, J. Narayanan, M.A. Karell, M. Xayavong, S. Kariuki, A.J. Dasilva, et al., Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR, Plos One 8 (2013) e56677.