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Padlock probe-mediated qRT-PCR for DNA computing answer determination

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Abstract

Padlock probe-mediated quantitative real time PCR (PLP-qRT-PCR) was adapted to quantify the abundance of sequential 10mer DNA sequences for use in DNA computing to identify optimal answers of traveling salesman problems. The protocol involves: (i) hybridization of a linear PLP with a target DNA sequence; (ii) PLP circularization through enzymatic ligation; and (iii) qRT-PCR amplification of the circularized PLP after removal of non-circularized templates. The linear PLP was designed to consist of two 10-mer sequence-detection arms at the 5' and 3' ends separated by a core sequence composed of universal PCR primers, and a qRT-PCR reporter binding site. Circularization of each PLP molecule is dependent upon hybridization with target sequence and high-fidelity ligation. Thus, the number of PLP circularized is determined by the abundance of target in solution. The amplification efficiency of the PLP was 98.7% within a 0.2 pg–20 ng linear detection range between thermal cycle threshold (C_t value) and target content. The C_t values derived from multiplex qRT-PCR upon three targets did not differ significantly from those obtained with singleplex assays. The protocol provides a highly sensitive and efficient means for the simultaneous quantification of multiple short nucleic acid sequences that has a wide range of applications in biotechnology.

Keywords

DNA computing; Padlock probe; Quantitative real-time PCR; TaqMan chemistry; Ordered node pair abundance; Traveling salesman problem

1 Introduction

DNA computing utilizes the hybridization of DNA molecules as a means to make computations (Adleman 1994; Lipton 1995). The ability of multiple sequences of DNA to hybridize in a solution simultaneously makes this approach particularly well-suited to solve computationally intense problems like simulating and predicting the emergent behavior of complex systems, optimizing business planning and production, targeting functional molecules in combinational chemistry, and the traveling salesman problem (TSP). The purpose of a TSP is to identify the most efficient tour through a network of arcs that visits a given set of nodes (cities) before returning to the starting point (Lee et al. 2003, 2004;

Tanaka et al. 2005; Spetzler et al. 2008). Xiong et al. (2009) recently solved a fully connected, asymmetric 15-city TSP, the largest problem solved to date, using DNA computing.

To solve a 15-city TSP, Xiong et al. (2009) designed 20mer DNA sequences that specifically encoded the nodes and arcs in the network, which were added to the reaction mixture in the presence of ligase. The sequence of each arc was capable of linking two node sequences in a specific order via hybridization of the last 10 bases of the prior node to the first 10 bases of the subsequent node. The efficiency of each arc in the network was encoded as the concentration of DNA for that arc using saturating concentrations of DNA for each node. Ligation of these sequences then formed covalently linked ordered node pairs (ONPs) that assembled into answer sequences representing tours through the network. When arc efficiency is encoded by the concentration of the arc DNA, the answer population is symmetrically distributed where the mode and mean correspond to the optimal answer. The probability of the formation of any one particular tour can be defined as the product of each arc in the path being traversed. Thus, for the tour (A, B, C, D, E) the probability was

$$P_{A,E} = P(A, B) * P(B, C) * P(C, D) * P(D, E)$$

The probability of any one connection was

$$P(A, B) = \frac{[AB]}{\sum [AX]}$$

where X is a member of the set of all vertices in the graph, the square brackets denote concentration. Thus, the larger the concentration of a particular node pair, the more likely it will be part of a tour.

The optimal answer of the 15-city TSP was identified by the ordered node pair abundance (ONPA) approach accomplished using Ligation Chain Reaction (LCR) (Spetzler et al. 2008; Xiong et al. 2009). In this approach, the DNA strands comprising the feasible answers of Hamiltonian circuits formed as the result of the computation were examined to identify those sequences of ordered node pairs in greatest abundance. Unfeasible answer sequences were removed by electrophoresis and magnetic bead separation prior to determination of the optimal answer. For each ordered node pair examined, probes were designed to hybridize to the 3'-end of the prior node and the 5'-end of the subsequent node in the ordered pair as well as a complimentary pair for use in LCR. Each pair of probes was then subjected to the same number of LCR cycles in the presence of an aliquot of answer sequences, and the relative abundance of LCR product was quantified by PAGE band intensity. This was repeated for every possible combination of ordered node pairs to identify the ones present in greatest abundance. Although this provided a clear determination of the optimal answer, the procedure required days to complete. In addition, the accuracy of the computation was limited by the ability to determine DNA abundance via bands in a gel, and by the fact that LCR is an end-point assay.

Other methods developed to determine answers from DNA computing include the use of a gradient PCR procedure that involved a series of PCR reactions with a variety of primer pair combinations to determine the solution to a 7-city directed Hamiltonian pathway problem (Adleman 1994). Answer determination has also been accomplished by DNA sequencing (Lee et al. 2004), or by the denaturation temperature gradient-polymerase chain reaction (DTG-PCR) associated with denaturing gradient gel electrophoresis (DGGE) and/or

temperature gradient gel electrophoresis (TGGE) (Yamamoto et al. 2002; Lee et al. 2003). These answer determination methods are time-consuming, and can not be used to solve several types of DNA computing problems including a TSP (Lee et al. 2003; Tanaka et al. 2005) because the discriminatory powers of DTG-PCR, DGGE, and TGGE are dependent on the diversity of oligonucleotide components, especially the G and C content (Riesner et al. 1992; Henco et al. 1994). In addition, the feasible answers of a TSP exist as a heterogeneous composite of DNA sequences that represent optimal and sub-optimal answers in a single aqueous solution. Since these answers contain the same composition of node sequences that differ only in the order in which the components were ligated when the answers were formed, neither DNA sequencing nor DGDC/TGDC can be used to identify the optimal the answer.

Quantitative real-time PCR (qRT-PCR) has proven to be a fast, precise and accurate method to determine the initial amount of DNA in a sample (Bustin et al. 2005; Valasek and Repa 2005). The advantages of this approach, which uses an increase in fluorescent signal to monitor increases of PCR amplicons, are due in part because it is not an end-point assay. Instead, the cycle at which a positive signal is first consistently detectable, termed the cycle threshold (C_t), is proportional to the initial content of a given template in the sample. Higher initial target contents give rise to earlier detectable increases in signal, resulting in a lower C_t value. Simultaneous amplifications of multiple DNA targets in a single reaction tube can be achieved by multiplex qRT-PCR, which maximizes throughput and decreases the time required to collect information. Recently, Ibrahim et al. (2008) reported a RT-PCR approach to readout answers to a Hamiltonian path problem. However, the protocol required multiple-combinations of RT-PCR amplifications and was ultimately dependent upon the use of silicon-based information processing to determine the answer.

Answer determination via qRT-PCR amplification of the entire length of TSP answer sequences is impossible. This is due to the intra-molecular heterogeneity of answers sharing the same starting and the ending nodes, which are the same limitations that eliminate the use of DTG-PCR. The application of qRT-PCR for the quantification of the short 20mer sequences using ONPA for answer determination of a TSP has also not been possible because standard PCR methodologies require two DNA primers that flank the region of the DNA sequence to be amplified. However, the recent use of padlock probes (PLPs) to diagnose plant pathogens by qRT-PCR (Szemes et al. 2005) may provide a possible solution to the problem of quantification of short DNA sequences required for DNA computing.

We now report the development of a PLP-qRT-PCR system capable of rapidly quantifying the abundance of short DNA sequences under the multiplex conditions required for the ONPA approach in DNA computing. Upon hybridization to its 20-mer target DNA, the PLP becomes circularized by ligation. This allows the abundance of adjacent short sequences in a DNA strand to be transformed into the copy number of circularized PLP that can be amplified by qRT-PCR. Our results show that the PLP-qRT-PCR assay combines sensitivity and high throughput efficiency of qRT-PCR with the high specificity of sequence-tagged hybridization and ligation in a manner that should significantly increase the speed and accuracy in which problems are solved by DNA computing.

2 Materials and methods

2.1 Preparation of target-specific padlock probes

Three 40-mer, synthesized oligo sequences designated BC, DE, and FG (Table 1) served as DNA targets to evaluate the PLP-qRT-PCR assay. These sequences correspond to those assigned to ordered node pairs BC, DE and FG in the 15-city TSP problem solved by DNA computing (Xiong et al. 2009). Each PLP consisted of one 55-mer core and two 10-mer

target-specific sequences that comprised the 5' and 3' arms located at ends of the core (Fig. 1). For BC, DE and FG targets, three 5'-arms of the PLP complementary to the last 10 bases of either sequence B, D, or F (designated B', D', and F'), and three 3'-arms (designated C'', E'', and G'') that were complementary to the first 10 bases of target sequences C, E, or G were created.

The core contained forward and reverse PCR primer-binding sequences (P_f 19-mer and P_r 21-mer), a restriction site, and a RT-PCR reporter-identifying sequence (RIS) known as a TaqMan spacer for use with a TaqMan-MGB® (Applied Biosystems) reporter dye. Three different core sequences were made so that the core could be used with TaqMan-MGB® reporter dyes FAM ($\lambda_{\max} = 518$ nm), VIC ($\lambda_{\max} = 554$ nm), and NED ($\lambda_{\max} = 580$ nm). The reporter dye ROX ($\lambda_{\max} = 610$ nm) was assigned by the vendor and served as internal reference dye. To assemble a PLP from core and arm components by ligation, the 3'-arm and core sequences were first phosphorylated in 30 μ l of reaction mixture containing phosphate reaction buffer, 10 nM oligonucleotide, 8.3 mM ATP, and 25 units of phosphonucleotide kinase. Prior to the reaction, the oligonucleotides were denatured at 70°C for 5 min, followed by a rapid sample transfer to ice-water. The reaction was conducted at 37°C for 30 min, followed by a 10 min inactivation at 80°C.

To ligate the PLP components, the sequences corresponding to the 5'-arm, phosphorylated core, and the phosphorylated 3'-arm were hybridized to complimentary-binding support Linkers 1 and 2, each of which was present in a final concentration of 20 nM. Ligation was conducted at 37°C for 30 min, followed by a 10 min inactivation at 80°C. The mixture was denatured at 92°C for 4 min, followed by cooling to 8°C at a rate of 2°C per min. Ligation was initiated by adding 1 μ l of 1 M DTT, 1 μ l of 250 mM ATP, and 7.5 Weiss U of T4 DNA ligase to a final volume of 20 μ l, and was incubated at 10°C for overnight. The 76-mer ligation product for each PLP made was purified by PAGE.

The three TaqMan-MGB® reporters, each with a different fluorescent dye and its own individual 15mer DNA sequence for identifying the corresponding template, were purchased from Applied Biosystems. All other oligonucleotide sequences were designed using Primer Express Version 2 for Windows (Applied Biosystems) and were synthesized by Invitrogen Inc.

2.2 Circularization of the PLP and the qRT-PCR assay

The PLP was circularized by denaturing and annealing the linear PLPs with the corresponding templates and the ligation reaction was performed at 10°C overnight. Unless stated otherwise, the reaction mixture contained 20 μ M of Oligo target, 20 pM of linear PLP, 50 mM DTT and 5 Weiss U of T4 DNA ligase (Fermentas) in a final volume of 20 μ l of ligation buffer. After ligation, 2 μ l of ligation product was added to 18 μ l of the exonuclease mixture that contained 10 mM Tris-HCl, pH 9.0, 5 mM MgCl₂, 0.1 mg per ml of BSA, 10 U Exonuclease I and 10 U Exonuclease III to remove any remaining linear PLP. The samples were incubated at 37°C for 2 h followed by inactivation at 65°C for 20 min.

qRT-PCR assays were performed in a 96-well, closed-plate using the AB 7500 Fast RT-PCR System (Applied Biosystems). Typically, qRT-PCR was performed in 20 μ l of qRT-PCR reaction mixture that contained 10 μ l of 2 $\times$ TaqMan® Fast Universal PCR Master Mix including the ROX fluorescent reporter as a passive reference, 900 nM of each of the P_f and P_r primers, one (singleplex assay) or three TaqMan-MGB® reporters (multiplex assay), and ~2 ng of circularized PLP unless indicated otherwise. Thermal cycling profiles for qRT-PCR included heating at 95°C for 20 s followed by 45 cycles with 95°C for 3 s and 60°C for 30 s.

During the qRT-PCR assay, PCR amplification of DNA copy number was monitored by quantitatively analyzing fluorescence emissions using an ABI prism sequence detector. The intensity of the VIC, NED, and FAM reporter dyes were measured against the ROX internal reference dye signal to normalize for non-PCR-related fluorescence fluctuations that occurred from well to well. The threshold cycle (C_t) represented the refractance cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated from PCR cycle 5–15.

2.3 Polyacrylamide gel electrophoresis (PAGE)

PCR amplified products (10 μ l) were profiled on 6% denaturing PAGE containing 8 M urea and 15% formamide at 95 V for about 3 h. The gels were visualized with UV light after staining with ethidium bromide (1 mg/ml) for 10 min. Sizes of the fragments were determined by comparison with mobility of a 20-bp DNA ladder (Bayou BioLabs).

3 Results

3.1 Steps in the PLP-qRT-PCR protocol

Figure 2 shows a denaturing PAGE gel of the purified padlock probes (PLPs) prepared as described in “Materials and methods”. The PLPs were designed to detect the specific DNA sequences for the BC, DE, and FG ordered pairs of DNA target sequences and contained the VIC, NED, and FAM qRT-PCR reporter-identifying sequences, respectively. The efficacy of a PLP to quantify the abundance of its respective target DNA sequence was determined using the sequential steps summarized in Fig. 3 for the BC-PLP. Each PLP was designed to hybridize to the last 10 bases of the preceding target sequence and the first 10 bases in the subsequent sequence of an ordered sequence pair of its target. The conformation of this hybridized complex is similar to that formed by a padlock probe (Nilsson et al. 1994). The hybridized PLP was circularized by ligase, after which the PLP was denatured from the target strand and exonucleases were added to eliminate any non-circularized PLP. The sequence encoding the *FokI* restriction site inserted at the interface of the P_f and P_r sequences in the PLP provided the option to linearize the circularized DNA by the addition of *FokI*. This positions the binding sites for P_f and P_r primers at the 5' and 3' ends.

3.2 Circularization of the linear PLP

Upon incubation with the Oligo target for the ordered BC sequence pair, the linear BC-PLP was circularized by hybridization and enzymatic ligation reactions when the terminal 3' hydroxyl and 5'-phosphate groups were juxtaposed on the target as in Fig. 3, steps 1–3. The circularization efficiency was evaluated by PAGE profiling after normal PCR amplification as shown in Fig. 4. In lanes 1–3, PCR amplification of the ligation products resulted in a major 75-bp DNA band. Since the first PCR cycle could occur only if the PLP had become circularized, the presence of the amplified 75-bp DNA band indicates that the circularization step was effective. It is noteworthy that two additional minor bands (~150-bp and ~220 bp) were also formed that were approximately 2 and 3-fold, respectively, the molecular size of the 75-mer PLP amplicons. When the circularized template was line-arized at the restriction site by *FokI* prior to amplification by PCR, the larger bands were absent and only the 75-bp DNA band was observed (Fig. 4, lanes 4–6). This confirmed that the 150 and 220 bp bands were the products of rolling-cycle amplification of the circularized amplicons.

3.3 Assay sensitivity and specificity

The sensitivity of the assay and linearity of the response was evaluated by establishment of a standard curve generated by qRT-PCR using serial dilutions of known target. Figure 5A shows a representative fluorescence amplification plot of the DE-PLP in six serial 10-fold

dilutions. The C_t values were determined in duplicate and plotted against the target content. The resulting standard curve of the DE target showed that the PCR amplification efficiency was 98.7% with a linear detection range between 0.2 pg and 20 ng that had a correlation of $R^2 > 0.99$ between the thermal cycle threshold and the template content (Fig. 5B). Similar correlations and PCR efficiencies were obtained from assays using the BC and FG targets (data not shown). The sensitivity of this assay was comparable to those of other qRT-PCR applications that were able to detect similar amounts of much longer DNA sequences isolated from plant pathogenic fungi (Ward and Bej 2006). No difference in sensitivity was detected when the *FokI* restriction step was omitted. Thus, the presence of some rolling circle events during qRT-PCR when circularized PLPs were used did not affect the quantification of target DNA.

The specificity of each PLP for its correct target was evaluated in Fig. 6 which shows the results of PLP-mediated qRT-PCR simultaneously monitored for VIC (circles), NED (squares), and FAM (triangles) fluorescence from each reaction vessel that contained equal amounts of the BC, DE and FG targets. Each reaction vessel contained a PLP specific for one target. The data for the BC VIC-PLP, DE NED-PLP, and FG FAM-PLP are shown in Fig. 6A–C, respectively. In each of these singleplex assays, the only consistent increase in fluorescent signal was associated with the qRT-PCR reporter-identifying sequence specific to the PLP present in the sample. This indicates that the increase in fluorescence was dependent upon hybridization and ligation of each PLP with its respective correct target.

3.4 Optimization of multiplex qRT-PCR

Figure 7 shows the fluorescence amplification plots as a function of cycle number from PLP-mediated multiplex qRT-PCR after factors that affect amplification efficiency in the qRT-PCR process were systematically adjusted to optimize the assay. In these multiplex experiments, equal amounts of the three target sequences were present in each reaction vessel as in Fig. 6. Each reaction vessel contained the BC VIC-PLP, DE NED-PLP, and FG FAM-PLP, but the fluorescence increase from only one TaqMan-MGB® reporter was monitored at a time. Optimization factors that were varied included the: (i) amount of enzyme used based on its efficiency and activity; (ii) pH; (iii) annealing temperature; (iv) concentrations of primer, Mg^{2+} , and dNTP; (v) primer specificities; and (vi) target content. The best conditions for multiplex qRT-PCR with consistent amplifications of all three PLPs were observed using 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 μ M primers, 0.06 ng/ μ l template, 0.2 mM each dNTP, and 0.04 U/ μ l *Taq* DNA polymerase.

The C_t values derived from the data of Figs. 6 and 7 are summarized in Table 2. These data show that under the optimized multiplex conditions, the amplification efficiency of each PLP was not affected by the presence of the other two PLPs and targets. Control samples with mismatched targets did not show a consistent increase in fluorescence intensity similar to that observed in Fig. 6 (data not shown). Thus, PLP-mediated qRT-PCR can provide quantitative information concerning the abundance of multiple DNA targets under multiplex conditions.

4 Discussion

The results presented here report the development of a PLP-qRT-PCR system capable of rapidly quantifying the abundance of short DNA sequences under the multiplex conditions required for the ONPA approach in DNA computing. This system provides several advances from that used previously for DNA computing including significantly improved sensitivity, accuracy, specificity, and high throughput efficiency as well as the ability to detect multiple

targets. These advances derive from the high-fidelity and specificity of sequence-tagged ligation as well as sensitivity and quantitative precision of qRT-PCR.

The 7500 RT-PCR system employed in these experiments uses 96-well plates. As a result, the 56 PLPs that are required to determine the optimal answer for an 8-city TSP can all use the same dye as long as they are tested in 56 different wells of the same plate. The 210 PLPs required to identify the optimal answer of a 15-city TSP can still be accomplished in a single 96-well plate using PLPs that contain two different dyes. In this latter multiplex situation, PLPs with different dyes that occupy the same well must be chosen that do not have a common target. To solve much larger problems, multiple 7500 RT-PCR instruments each containing a 96-well plate could be run simultaneously as long as some of the wells in each plate share a few common PLPs to correct for differences in the instrumentation. The design of PLPs described in Fig. 1 that enables their assembly from stock arm and core components simplifies the task of making all of the PLPs with the appropriate dye for multiplexing.

By circularizing the linear PLP via template-tagged ligation, which is quantitatively related to the initial content of a target, the target content used in the determination was transformed into the copy number of the circularized PLPs. Thus, only the circularized PLP molecules, and not the original DNA targets, are amplified by qRT-PCR. Other DNA target amplification techniques that are used to detect and quantify DNA like PCR usually amplify the original DNA targets. Use of the original DNA targets for amplification can cause serious errors in quantifying the abundance of a sample due to differences in amplification efficiency of different DNA sequences and the possibility of nonspecific primer-target interactions (Nilsson et al. 1994; Lievens et al. 2003; Szemes et al. 2005).

The multiplex qRT-PCR results presented here were carried out using a pair of PCR primers that were universal to all of the PLPs. Optimization of multiplex qRT-PCR is a common challenge that is mainly attributed to the involvement of multiple PCR primer pairs because the first few rounds of thermal cycling have a substantial effect on the overall sensitivity and specificity of qRT-PCR (Burgos et al. 2002). The presence of more than one primer pair in the multiplex has been found to significantly increase the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (Szemes et al. 2005). The use of the same pair of PCR primers by all PLPs eliminates these nonspecific interactions that are often responsible for serious difficulties in the optimization of multiplex qRT-PCR.

Short amplicons (70–100 bp) have been shown to provide greater C_t reproducibility with qRT-PCR than observed from longer amplicons (Burgos et al. 2002). The TaqMan® reporters used in the PLPs reported here were all MGB-associated such that they were 10–12 bases shorter than the standard TaqMan reporters. However, the melting temperatures of the two types of TaqMan reporters are comparable (~62–68°C). Consequently, the entire amplicon derived from a PLP was only 76-bp long including the 5' and 3' arms, the two PCR primer-binding sequences (P_f and P_r), and the qRT-PCR reporter-identifying sequence. This ensured consistent detection of fluorescent signals through target-dependent circularization of the PLP.

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Fig. 1.

Assembly of padlock probes (PLPs) from stock arm and core components. The core contained forward and reverse PCR primer-binding sequences (P_f 19-mer and P_r 21-mer), a *FokI* restriction site (R), and the RT-PCR reporter-identifying sequence (RIS) known as a TaqMan spacer for use with either the VIC, NED, or FAM TaqMan-MGB® (Applied Biosystems) reporter dye. Assembly of the PLP is shown that contains the RIS for VIC and will hybridize to the BC target where the target DNA contains the sequence in which B precedes C (BC), designated the BC-VIC-PLP. This PLP contains the 5'-arm complementary to the last 10 bases of target sequence B (B') and the 3'-arm complementary to the first 10 bases of target sequence C (C''). The core becomes ligated to the 3' and 5'-arms following hybridization with disposable linkers. After ligation the linkers are removed by purification of the assembled PLPs using denaturing PAGE. Sequences of the components are provided in Table 1. See "Materials and methods" for details

Fig. 2. Denaturing PAGE profile of ligation products in the assembly of the BC-VIC-PLP (*lane 1*), the DE-NED-PLP (*lane 2*), and FG-FAM-PLP (*lane 3*). See “Materials and methods” for experimental details. A 20-mer DNA ladder (*lane M*), where the 100mer was the brightest band (*arrow*), was used as a molecular size reference

Fig. 3.

PLP-dependent sample preparation for determination of target copy number by PLP-mediated qRT-PCR. (1) Hybridization of the PLP to 20 consecutive bases of the target sequence. (2) Circularization of the hybridized PLP by T4 ligase. (3) Elimination of any linear nucleic acid by Exonuclease I and III after denaturation of the circularized PLP from target. (4) Optional restriction by *FokI* of the circularized PLP to create a linear sequence where the forward (P_F) and reverse (P_R) PCR primers are at the 5' and 3' ends. (5) Use of qRT-PCR to quantify PLP abundance upon addition of the TaqMan-MGB® reporter, PCR primers and nucleotides

Fig. 4. PAGE profile of PCR products of circularized BC-PLP (*lanes 1 and 4*), DE-PLP (*lanes 2 and 5*), and FG-PLP (*lanes 3 and 6*) before (*lanes 1–3*) and after (*lanes 4–6*) digestion with the *FokI* restriction enzyme. A 20-mer DNA ladder (*lane M*) where the 100mer was the brightest band was used as a molecular size reference

Fig. 5.

Standard curve for the amount of a short target DNA sequence quantified by PLP-mediated qRT-PCR. **A** Fluorescence intensities were monitored during PLP-qRT-PCR using 20 pM of the DE-NED-PLP and 0.25 μ M of the NED TaqMan-MGB® reporter in the presence of 2×10^1 ng (filled circle), 2×10^0 ng (filled square), 2×10^{-1} ng (filled inverted triangle), 2×10^{-2} ng (open square), 2×10^{-3} ng (open circle), 2×10^{-4} ng (open triangle), 2×10^{-5} ng (open diamond) of the DE DNA target. See “Materials and methods” for experimental details. **B** The C_t value of the PLP-qRT-PCR from (A) as a function of the amount of target

Fig. 6.

Target specificity of singleplex PLP-mediated qRT-PCR. Each reaction vessel contained 20 μ M of either the BC (**A**), or DE (**B**) or FG (**C**) DNA targets. Each assay contained the BC-VIC-PLP, DE-NED-PLP, and FG-FAM-PLP along with TaqMan-MGB® reporters, VIC (*open circle*), NED (*open square*) and FAM (*open triangle*). Fluorescence intensities raised during PCR amplifications were simultaneously monitored at 520, 550 and 580 nm wavelengths specific for VIC, NED and FAM, respectively. Each reaction was measured in triplicate. See “Materials and methods” for experimental details

Fig. 7.

Multiplex PLP-mediated qRT-PCR following optimization of conditions show consistent signal amplification (*solid circles*) that are almost identical to those of the singleplex assay from Fig. 6 (*open circles*). For the multiplex assays, each reaction vessel contained 20 μ M of each of the BC, DE and FG DNA targets as well as the BC-VIC-PLP, DE-NED-PLP, and the FG-FAM-PLP, and the TaqMan-MGB® reporters for VIC, NED, and FAM. See “Materials and methods” for experimental details. Fluorescence intensities were monitored at wavelengths specific for detection of fluorescence from VIC (**A**), NED (**B**) and FAM (**C**). Each reaction was measured in triplicate for multiplex samples (*solid circles*) or singleplex samples from Fig. 6 (*open circles*)

Table 1**Oligonucleotide sequences used for PLP-qRT-PCR**

VIC-TaqMan® Spacer	CGCGGATGTCGGTCAGCCGAGTCTACCCAGCGCGCCAC TACAGCTGCAGCAGTCG
NED-TaqMan® spacer	CGCGGATGTCGGTCAGCCGAGTCTACCCAGCGCGCCACTAT CGCCATCAGGCAGC
FAM-TaqMan® spacer	CGCGGATGTCGGTCAGCCGAGTCTACCCAGCGCGCCACAA CCGTGACTTCTGCCA
<i>Target sequences (20-mer + 20-mer)</i>	
BC	TTTACGTCTACCATATCTATATAGCAACACTACATATGTC
DE	TCGACTAATTTCGTACTTATACCTGATACAAGTACTAAGTA
FG	GCGTAAGGATATTTATACAAGTTTGTTTAGTCCATCATT
<i>TaqMan-M GB® reporters</i>	
VIC	VIC-CAGCTGCAGCAGTCG-MGBNFQ
NED	NED-TCGCCATCAGGCAGC-MGBNFQ
FAM	6FAM-CCGTGACTTCTGCCA-MGBNFQ
<i>ARMS for target recognition</i>	
5'-B'	ATAGATATGG
5'-D'	TATAAGTACG
5'-F'	TTGTATAAAT
3'-C''	GTGTTGCTAT
3'-E''	TTGTATCAGG
3'-G''	CTAAACAAAC
<i>Linkers for PLP assembly</i>	
BC Linker 1	GACATCCGCGCCATATCTAT
DE Linker 1	GACATCCGCGATTATACAA
FG Linker 1	GACATCCGCGCGCGGATGTC
BC Linker 2	ATAGCAACACCGACTGCTGC
DE Linker 2	CCTGATACAAGCTGCCTGAT
FG Linker 2	GTTTGTTTAGTGGCAGAAAGT
<i>qRT-PCR primers</i>	
P _r primer	CTCGGCTGACCGACATCCGCG
P _f primer	AGTCTACCCAGCGCGCCAC

Table 2

Amplification efficiencies of singleplex versus multiplex PLP-mediated qRT-PCR

PLP	TaqMan-MGB® reporter	C _t	
		Singleplex ^a	Multiplex ^b
BC	VIC	15.5	16.0
DE	NED	15.8	16.0
FG	FAM	11.8	12.0

^aDetermined from the data in Fig. 6^bDetermined from the data in Fig. 7