

Reports

Polymerase chain displacement reaction

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Quantitative PCR assays are now the standard method for viral diagnostics. These assays must be specific, as well as sensitive, to detect the potentially low starting copy number of viral genomic material. We describe a new technique, polymerase chain displacement reaction (PCDR), which uses multiple nested primers in a rapid, capped, one-tube reaction that increases the sensitivity of normal quantitative PCR (qPCR) assays. Sensitivity was increased by approximately 10-fold in a proof-of-principle test on dengue virus sequence. In PCDR, when extension occurs from the outer primer, it displaces the extension strand produced from the inner primer by utilizing a polymerase that has strand displacement activity. This allows a greater than 2-fold increase of amplification product for each amplification cycle and therefore increased sensitivity and speed over conventional PCR. Increased sensitivity in PCDR would be useful in nucleic acid detection for viral diagnostics.

PCR is a key technique used in molecular biology to amplify specific regions of DNA; the amplified product is traditionally detected by gel electrophoresis. The emergence of quantitative PCR (qPCR) using fluorescent probes or dyes now allows amplification and analysis simultaneously, conferring rapid detection and quantification of target DNA. qPCR also enables higher throughput with a reduced risk of cross-contamination, as there are no post-PCR manipulations. The amplified product in qPCR can be detected by either nonspecific DNA binding dyes, for example SYBR green, which binds to any double-stranded DNA present, or by probes that hybridize to specific target sequences (1–4).

In virus detection, qPCR has now become the standard technique, as it is quick, sensitive, and easier to perform than the more traditional method of culturing the virus in cell lines (5). Virus culture can take up to 2 weeks, depending on the virus, and the result can be hindered by the presence of antibodies in the patient's serum. qPCR is also being used over serological analysis, which detects antibodies specific to the virus in a patient's serum. Serological analysis prevents early, rapid diagnosis, as antibodies are generally only produced 6–14 days after infection. Serological analysis is also not possible when trying to detect the presence of virus in samples that are not human serum, for example in arboviral vectors such as mosquitoes. Nucleic acid detection with qPCR has a further advantage of quantifying the

copy number of virus in a patient's plasma, which can be vital for monitoring some diseases (6).

Conventional PCR uses a pair of primers and therefore produces a maximum of a 2-fold increase in amplicon per amplification cycle. We have developed a modification to PCR that uses more than one pair of primers; we call this variant polymerase chain displacement reaction (PCDR). Nested primers are designed that flank the region of interest; when the outer and inner primers are extended, the extended strand of the outer primer causes displacement of the extended strand produced from the inner primer (Figure 1). The polymerase used in PCDR is a modified *Taq* DNA polymerase that possesses strand displacement activity and lacks 5' to 3' exonuclease activity; therefore degradation of the inner primer extension product does not occur. In PCDR, we use a dual-labeled probe, which has a quencher at the 3' end and a fluorophore at the 5' end. When there is no target present, the two ends of the probe come close to one another, in a random coil, resulting in the fluorophore being quenched; once there is template for the probe to bind to, the quencher and fluorophore are separated, allowing fluorescence to be generated (7). PCDR allows more than two amplicons to be produced after each amplification cycle and therefore greater sensitivity and increased speed of assay. An increase in the sensitivity of amplification assays would be beneficial in diagnostic uses for virus detection where there is a low

starting copy number or where sensitivity is lost by PCR inhibitors present in crude extracts (8).

Another advantage of using multiple primers in PCDR for viral detection is due to the high frequency of mutations that occurs in viral genomes, producing multiple subtypes with a high level of genetic variation within each virus (9,10). Primers are very specific; therefore detection via PCR is vulnerable to false negatives due to genome sequence diversity. In viral diagnostics, PCR primers need to be designed so that all subtypes are identified and new subtypes could possibly be recognized. In one study, four commercial quantitative viral load assays for HIV were tested with a panel of diverse patient samples taken from multiple regions and as few as 88.6% were positively detected from 97 plasma samples. These false negatives were found to be due to mutations in the primer or probe binding sites (6). A single pair of primers is more susceptible to false negatives due to these mutations, whereas if four primers are used together, as in PCDR, it is much less likely that mutations would occur in both primer binding sites and therefore amplification would still occur.

To determine if sensitivity and speed could be improved by PCDR in viral detection, we developed an assay to identify the presence of dengue virus sequence. Dengue virus (DENV) is a positive strand RNA flavivirus that has four distinct serotypes (DENV1–4) and is the causative agent of dengue fever (11). Dengue is

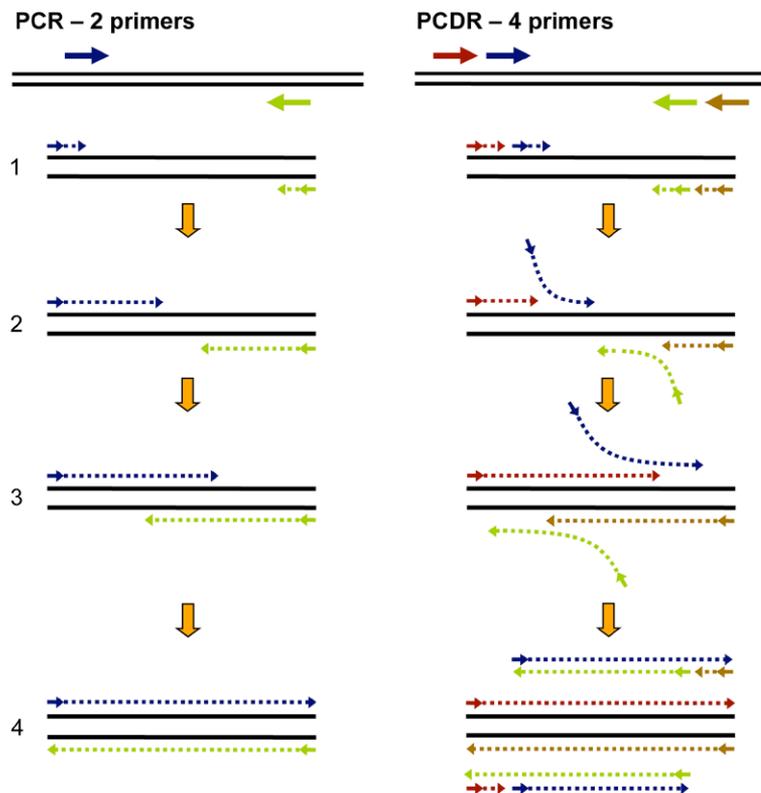


Figure 1. Schematic showing the mechanism of PCDR. Conventional PCR uses two primers, and therefore the maximum increase of amplicon per amplification cycle is two. PCDR uses more than one pair of primers. When the outer primer is extended, it causes displacement of the extension strand produced from the inner primer. A polymerase that possesses strand displacement activity and that lacks 5' to 3' exonuclease activity is used to prevent stalling of the extension of the outer primer and degradation of the inner primer extension strand. This allows greater than two amplicons to be produced after each amplification cycle.

a mosquito-borne disease that is endemic in more than 100 countries, with an incidence that is rapidly increasing (12–15). While dengue fever presents with flu-like symptoms, this can progress to dengue hemorrhagic fever, which can be fatal. This usually occurs if the patient is infected with a second DENV serotype. PCR assays have been developed for the detection of dengue viral RNA. Some of these assays are capable of serotyping and/or able to quantify the viral load, which may be an indicator of disease severity (16). Only one PCR assay for DENV has been commercialized to date (RealArt; artus/Qiagen, Valencia, CA, USA) (17). The other in-house assays developed include nested reverse transcriptase PCR, where the result is visualized by gel electrophoresis (18–20); however this is not suitable for high-throughput diagnostics, and therefore multiplex quantitative assays have also been designed, including one-step quanti-

tative reverse transcriptase and multiplex quantitative assays (21–24).

We have developed a quantitative PCDR assay that uses pan-dengue primers to detect dengue virus sequence.

Materials and methods

Primers and probe design

PCR primers and probe were synthesized by Eurogentec (Seraing, Belgium). Their sequences are shown in Table 1, where they bind in the NC_001475 sequence, and their relative positions in Figure 2A. Generic pan-dengue primers, which amplify all four dengue serotypes, were used with a dengue 3 serotype-specific probe. The pan-dengue primers used are longer than normal PCR primers, so that all four dengue serotypes could be amplified despite some sequence variation.

Table 1. Sequences of primers and probes used in PCR assays.

Primer and probe	Sequence	5' nucleotide position
Den F2	5'-GGGAGGCCATAAACCATGGAAGCTGTACGC-3'	10440
Den F3	5'-GCCACGCTTTGAGTAAACTGTGCTGCCTGTAGCTC-3'	10384
Den R1b	5'-CTTATCTCTCCTTAACCTCTAGTCCTT-3'	10590
Den R2	5'-GGTCTTTCCCAGCGTCAATATGCTGT-3'	10626
Den3-Cy5 probe	5'-Cy5-CCCAGCACTGAGGGAAGCTGT-BHQ2-3'	10530

5' Nucleotide position of primer/probe in the DENV3 NC_001475 sequence.
Cy5, Cyanine 5; BHQ2, black hole quencher 2.

Preparation of DNA constructs for template DNA

A plasmid construct, pMA-T, containing a 395-nucleotide fragment of the DENV3 sequence was synthesized by GeneArt (Life Technologies, Paisley, UK); this includes nucleotides 10312–10707 from the published sequence (NC_001475), which is located in the 3' untranslated region (3' UTR) of the virus. The plasmid was digested with restriction enzymes, which have sites on either side of the DENV3 sequence, producing template DNA in a linear form. Tenfold dilutions of the digested plasmid were used in the quantitative assays spiked with 10 ng/μL of *Aedes aegypti* mosquito pupae RNA/DNA.

Amplification reactions

All amplification reactions were carried out on a T3000 Thermocycler (Biometra, Goettingen, Germany). Thermocycler conditions consisted of an initial denaturation 95°C for 2 min, and 34 cycles of 95°C for 5 s, 55°C or 15 s, 72°C for 15 s, and a final elongation of 72°C for 1 min. Eighteen microliters of the reaction were run on a 1.5% agarose gel and visualized by ethidium bromide staining. For sequencing of the amplicons, amplification product was separated by electrophoresis and gel purified, this was cloned into pJET1.2 (Fermentas, St. Leon-Rot, Germany) and transformed into XL-10 Gold cells (Stratagene, Santa Clara, CA, USA). Individual colonies were screened using primers that flank the cloning site, and the resulting PCR product was purified and sequenced by GATC (Konstanz, Germany).

Real-time amplification procedure

All qPCRs were performed using a Mx3005P (Stratagene) and analyzed with MxPro software (Stratagene). Assay components were optimized and consisted of 1× PCDR master mix (GeneFirst, Oxford, UK), 200 nM F2 primer, 60 nM F3 primer, 200 nM R1b primer, 100 nM R2 primer, and 200 nM Den3-Cy5 probe. Total reaction volume was 25 μL. The conditions consisted of an initial denaturation 95°C for 9 min and 45 cycles of 95°C for 6 s, 55°C for 30 s, and 72°C for 30 s. Assays using TaqMan Master Mix consisted of 1× TaqMan Master Mix (Applied Biosystems, Carlsbad, CA, USA), 200 nM F2 primer, 200 nM R1b primer, and 200 nM Den3-Cy5 probe. The conditions for qPCR using the TaqMan Master Mix consisted of an initial denaturation 95°C for 9 min and 45 cycles of 95°C for 6 s, 55°C for 30 s, 60°C for 20 s, and 72°C for 20 s. Reactions were carried out in clear plates and sealed with flat lid strips. All reactions were performed in triplicate or were done two or three times in separate experiments. Water was used for the no template control (NTC).

Intrathoracic injection of *Aedes aegypti* mosquitoes

Five-day-old adult, female *Aedes aegypti* RexD mosquitoes were intrathoracically inoculated

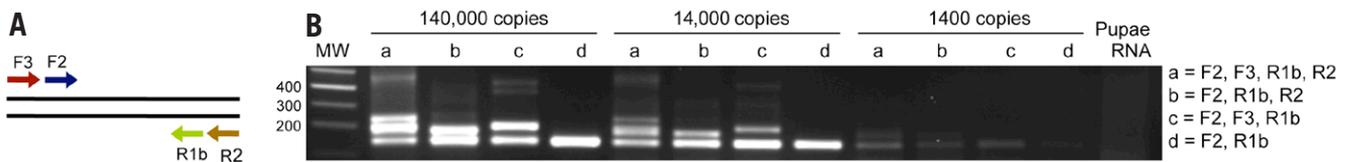


Figure 2. Comparison of PCDR with PCR. (A) Schematic showing the relative positions of PCR primers used in PCDR. (B) Amplification reactions on serial dilutions of synthetic plasmid containing DENV3 sequence using four primers F2/F3/R1b/R2 (a); three primers F2/R1b/R2 (b) and F2/F3/R1b (c); or two primers F2/R1b (d) were carried out for 34 cycles as detailed in Materials and methods section, and the products were analyzed by gel electrophoresis and ethidium bromide staining. Reactions with four primers produced multiple products and increased sensitivity. MW, molecular weight marker (SmartLadder; Eurogentec, Liège, Belgium).

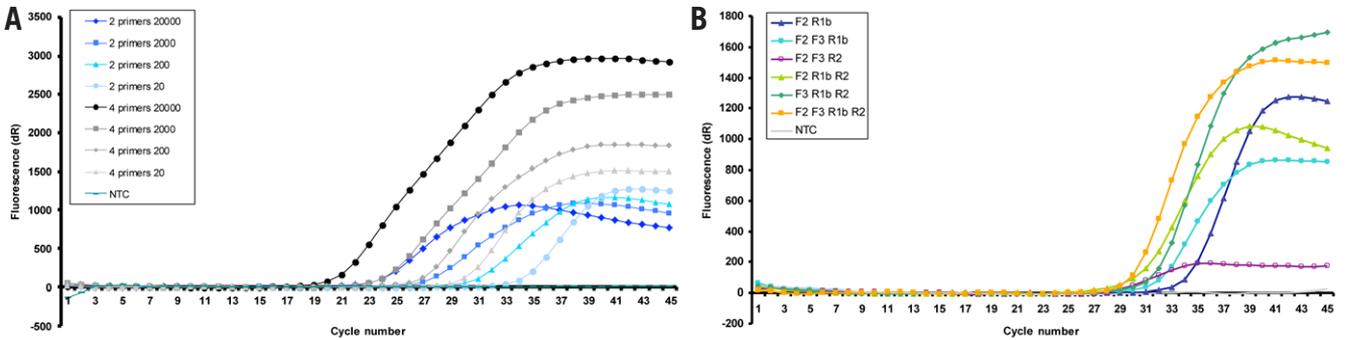


Figure 3. Increased sensitivity of quantitative PCDR when using four primers. (A) Amplification reactions were carried out using PCDR master mix with 20,000, 2000, 200, or 20 copies of DENV3 template DNA, Den3-Cy5 probe, and either four primers (F2, F3, R1b, and R2) or the inner two primers (F2 and R1b) as detailed in the Materials and methods section. (B) Amplifications were carried out using PCDR master mix with 20 copies of template DNA; Den3-Cy5 probe; and primers F2/F3/R1b/R2, F3/R1b/R2, F2/R1b/R2, F2/F3/R2, F2/F3/R1b, or F2/R1b; as described in the Materials and methods section. Fluorescence curves are an average from three independent experiments. NTC, no template control. Note that the low fluorescence signal from the “F2 F3 R2” primer combination on 20 copies of template is poorly understood and is possibly due to production of higher amounts of primer dimer.

with 4×10^2 pfu DENV3 (MX BC285/97). One week after inoculation, infection rates were confirmed by an immunofluorescence assay (IFA) on head squash smears using 5D4–11 MAb specific for DENV3. Mosquitoes were placed in RNA_{later} (Life Technologies) and stored at -20°C .

RNA extraction and cDNA synthesis

Total RNA was extracted from mosquitoes with TRIzol (Life Technologies) according to the manufacturer’s instructions. Mosquitoes were homogenized using a plastic pestle. RNA was checked for quality by gel analysis and quantity by spectrometry using GeneQuant II (GE Healthcare, Little Chalfont, UK) and stored at -80°C . A maximum of 5 μg RNA was converted to cDNA using SuperScript II Reverse Transcriptase (Life Technologies) with a mixture of R1b and R2 as the first strand primer, according to the manufacturer’s instructions. cDNA was treated with RNaseH (Life Technologies), stored at -20°C , and 1 μL cDNA was used in subsequent PCRs.

Results and discussion

Comparison of PCDR with PCR in gel-based assays

Conventional PCR only uses two primers in each reaction, in comparison to PCDR with multiple primers. To compare the sensitivity of PCR with PCDR, amplifications were carried out using four, three, or two of the pan-dengue

primers with 140,000, 14,000, or 1400 copies of DENV3 template DNA, and the amplification products visualized by gel electrophoresis (Figure 2B). Only one amplification product was produced with two primers, but the use of three or four primers produced two or three PCR products, respectively, showing that amplicons were produced using all the primers available. In the reaction with 1400 copies of template DNA using two primers, very little PCR product was produced. However, using three or four primers in PCDR, there was a clear increase in the amount of product, with four primers being more sensitive than three. The amplification products were sequenced to confirm specificity (i.e., that using multiple primers in PCDR did not produce any off-target amplicons). Sequencing confirmed that only the specific products were amplified; furthermore, all four predicted PCDR products were indeed amplified (data not shown). Although Figure 2B shows some higher molecular weight products with PCDR (e.g., lane 2, ~ 450 bp), these were sequenced and found to contain only the nominal sequence and are therefore presumably oligomers of the predicted products.

Comparison of PCDR with PCR using PCDR enzyme for quantitative assays

To determine if PCDR can improve the sensitivity of quantitative assays, the DENV3 specific probe was used in combination with four, three, or two of the pan-dengue primers. Before doing this, we first tested if the single

primer pairs in the four different combinations behaved differently. The result revealed that there was no significant difference in the quantification cycle (C_q) value or sensitivity for each primer pair (Table 2 and Supplementary Table S1). To compare PCDR and PCR, we chose the inner-most primer pair for the PCR. The sensitivity of each primer set was determined using 10-fold dilutions of DENV3 template DNA (Table 2 and Supplementary Table S1). Amplification curves for all dilutions are shown in Figure 3A, comparing four-primer PCDR with two-primer PCR; amplification curves of all primer sets for the reaction with 20 copies of templates are shown in Figure 3B. These results show that both lower C_q values and much improved fluorescence curves were achieved in four-primer PCDR in comparison to two-primer PCR for all dilutions tested. The C_q values were reduced by three cycles when using four primers instead of two. Efficiency was calculated by comparing C_q number against starting copy number and determining the slope of the graph produced. An efficiency of over 100% means a greater than 2-fold increase in amplicon per amplification cycle. The assays using four primers had an efficiency of around 107% (average from data of Tables 2 and 4); this is in comparison to 103% (average from data of Table 2 and Supplementary Table S1) when two primers were used. Although the total concentration of primer present was increased in the four-primer PCDR reaction, when the primer concentration was doubled in

Table 2. Comparison of quantitative PCDR and PCR.

Copy number	Cq number			
	Four primers F2, F3, R1b, R2	Three primers F2, R1b, R2	Three primers F2, F3, R1b	Two primers F2, R1b
20,000	20.47 ± 0.66	21.86 ± 0.62	22.21 ± 0.33	24.04 ± 0.69
2000	23.95 ± 0.89	25.12 ± 0.3	25.56 ± 0.59	27.76 ± 1.13
200	26.84 ± 0.71	28.58 ± 0.79	28.85 ± 0.67	31.07 ± 0.99
20	29.97 ± 0.54	31.66 ± 0.23	32.31 ± 0.74	34.17 ± 0.56
Efficiency (%)	108.2	101.5	110.4	98.1
R ²	1.00	1.00	1.00	1.00

Amplifications were carried out using PCDR master mix with 10-fold dilutions of DENV3 template DNA, Den3-Cy5 probe, and primers: F2/F3/R1b/R2; F2/R1b/R2; F2/F3/R1b; or F2/R1b as described in the Materials and methods section. Cq values and efficiencies for each primer combination are shown. Cq values are an average ± SD from three independent experiment; intra- and interassay coefficients of variation were 1.2%–4.08%. The slope and y intercept of the standard curves are: F2/F3/R1b/R2 = slope -3.14 and intercept 36.30; F2/R1b/R2 = slope -3.29 and intercept 38.31; F2/F3/R1b = slope -3.36 and intercept 38.99; F2/R1b = slope -3.37 and intercept 41.05. No Cq values were obtained for NTC. A lower Cq value was achieved with four primers in comparison to two.

Table 3. Comparison of quantitative PCDR and hydrolysis probe-based PCR.

Copy number	Cq number for PCDR		Cq number for TaqMan	
	Four primers F2, F3, R1b, R2	Two primers F2, R1b	Two primers F2, R1b	Two primers F2, R1b
20,000	20.27 ± 1.45	23.30 ± 1.17	23.30 ± 1.17	23.30 ± 1.17
2000	23.53 ± 1.13	26.65 ± 1.22	26.65 ± 1.22	26.65 ± 1.22
200	26.64 ± 1.20	30.56 ± 1.15	30.56 ± 1.15	30.56 ± 1.15
20	29.82 ± 0.91	33.91 ± 1.29	33.91 ± 1.29	33.91 ± 1.29
Efficiency (%)	106.5	90.4	90.4	90.4
R ²	0.99	1.00	1.00	1.00

Amplifications were carried out using PCDR master mix or TaqMan Master Mix with 10-fold dilutions of DENV3 template DNA, Den3-Cy5 probe, and primers: F2/F3/R1b/R2 or F2/R1b, as detailed in the Materials and methods section. Cq values and efficiencies for each primer concentration are shown. Cq values are an average ± SD from three independent experiments, with triplicates within each experiment; intra- and interassay coefficients of variation were 0–1.54% and 2.76–4.59%, respectively. F2/F3/R1b/R2 = slope -3.18 and intercept 36.18; F2/R1b = slope -3.58 and intercept 41.12. No Cq values were obtained for NTC.

a two-primer PCR, there was no change in Cq or efficiency (data not shown), showing that the lower Cq value in PCDR was not simply due to an increase in total primer concentration. Although the slight increase of efficiency is not likely to be of practical importance in most contexts, the consistently lower Cq value would mean increased sensitivity and speed of reaction.

Comparison of PCDR with hydrolysis probe-based PCR assays

PCR does not normally use more than one pair of primers to amplify the same DNA region. This is because the DNA polymerase used in normal PCR contains 5' to 3' exonuclease activity. Therefore, if a reaction contained nested primers for the same strand of a target sequence, the inner extension strand would be degraded due to exonuclease activity of the polymerase. Conventional PCR containing multiple nested primers would therefore not increase PCR efficiency or sensitivity. The hydrolysis probe-based qPCR relies on the 5' to 3' exonuclease activity of Taq DNA polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection. To compare PCDR and hydrolysis probe-based qPCR, we performed a four-primer PCDR assay and two-primer hydrolysis probe (TaqMan) PCR assay. We used TaqMan Master Mix from Applied Biosystems; this contains AmpliTaq Gold DNA polymerase, which has 5' to 3' exonuclease activity. qPCR was carried out using a DENV3 detection probe with four of the pan-dengue primers and PCDR master mix or two of the primers and the TaqMan Master

Mix. We first tested the four different combinations of primer pairs from two forward primers and two reverse primers in the hydrolysis probe-based qPCRs. As expected, the innermost pair of primers worked best (data not shown) and was used for comparison with PCDR. The sensitivity of PCDR and hydrolysis probe-based qPCR was determined using 10-fold dilutions of the DENV3 template DNA (Table 3). Representative amplification curves for 20 copies of template DNA in PCDR and hydrolysis probe-based qPCR are shown in Figure 4. The PCDR assays achieved consistently lower Cq values than the hydrolysis probe-based qPCR at all dilutions. At the lowest amount of 20 copies, the Cq value was 29.82 for PCDR using four primers compared with 33.91 for the hydrolysis probe-based qPCR.

Quantitative PCDR on mosquitoes infected with DENV3

To test whether PCDR improved the sensitivity of detecting actual dengue viral samples, RexD *Aedes aegypti* mosquitoes were infected with DENV3 virus by intrathoracic injection, and infection levels were confirmed by immunofluorescence assay on head squashes. RNA was extracted and converted to cDNA. The qPCRs were performed on undiluted and serially diluted cDNA (Table 4). PCDR showed increased efficiency over both two-primer assays, with improved Cq values, particularly for the reactions with a lower template concentration. Sequencing of the PCDR amplicons from these mosquito samples showed that no off-target amplicons were produced (data not shown).

Increased sensitivity of PCRs has been achieved previously by using nested primers.

For example, nested PCR has been incorporated into quantitative assays for the detection of *Mycobacterium tuberculosis* (25); however two separate rounds of PCR are required, which increases the run time and the risk of cross-contamination.

In this report, we describe PCDR, a novel technique that increases the sensitivity of amplification, similar to the use of nested primers, but in a single, closed-tube reaction. This decreases the chance of contamination and reduces the run time to equivalent to or even less than normal qPCR, while using conventional qPCR platforms. In these proof-of-principle experiments, we were able successfully to amplify and detect dengue virus sequences from various sources and types of material; however the performance of PCDR in other contexts remains to be assessed.

The increased sensitivity obtained by PCDR would be useful in assays with low template DNA, for example in viral diagnostics. Using multiple primers here would also have an advantage over conventional PCR for detecting all variants, helping to overcome the problem of template variation due to the high mutation rate of many viruses. False negatives in viral diagnostics have already been reported due to mutations in the primer/probe hybridization sites (26), and diagnostic kits should ideally be able to cope with these changes without compromising on specificity. However, in this context, PCDR only uses a single probe, and therefore this method could also be prone to false negatives due to mutations in the probe-hybridization site. PCDR could also be utilized in multiplex format to determine different serotypes of the same species, in which the probes are designed to specifically bind to each serotype.

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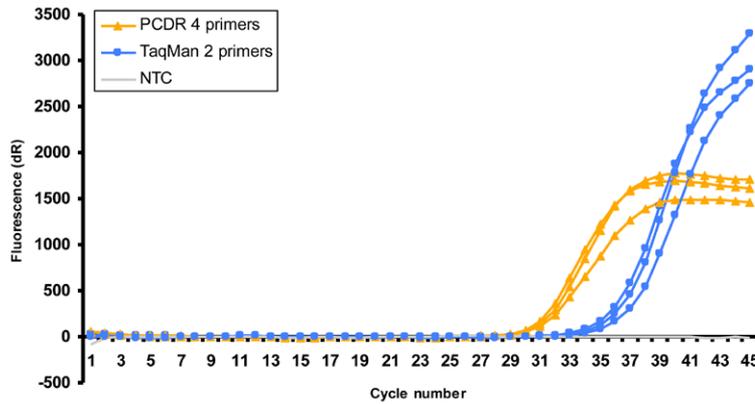


Figure 4. Comparison of quantitative PCDR and hydrolysis probe-based PCR. Amplifications were carried out using PCDR master mix or TaqMan Master Mix with 20 copies of DENV3 template DNA, Den3-Cy5 probe, and primers F2, F3, R1b, and R2 with the PCDR master mix; or F2 and R1b with the TaqMan Master Mix; as described in the Materials and methods section. Triplicate samples are shown. NTC, no template control. PCDR was more sensitive than hydrolysis probe-based PCR.

Table 4. Comparison of quantitative PCDR and hydrolysis probe-based PCR on mosquitoes infected with DENV3.

Dilution	Cq number		
	PCDR Two primers F2, R1b	PCDR Four primers F3, F2, R1b, R2	TaqMan Two primers F2, R1b
1/10 ¹	18.57 ± 0.60	15.88 ± 0.25	16.15 ± 0.27
1/10 ³	26.13 ± 0.53	22.78 ± 0.39	26.04 ± 0.78
1/10 ⁴	29.48 ± 0.56	26.45 ± 0.36	28.86 ± 0.27
1/10 ⁵	32.93 ± 0.34	29.66 ± 0.15	32.13 ± 0.47
1/10 ⁶	35.18 ± 0.51	32.28 ± 1.03	35.90 ± 0.36
Efficiency (%)	98.0	100.4	81.4
R ²	0.99	1.00	0.99

Amplifications were carried out using PCDR master mix or TaqMan Master Mix with 10-/100-fold dilutions of cDNA from mosquitoes infected with DENV3, Den3-Cy5 probe, and primers: F2/R1b or F2/F3/R1b/R2, as described in the Materials and methods section. Cq values and efficiencies for each primer combination are shown. Cq values are an average ± sd from three independent experiments, with triplicates within each experiment; intra- and interassay coefficients of variation were 0.1%–1.5% and 0.7%–2.8%, respectively. No Cq values were obtained for NTC. PCDR F2/R1b = slope -3.37 and intercept 35.87; PCDR F2/F3/R1b/R2 = slope -3.33 and intercept 32.74; TaqMan F2/R1b = slope -3.87 and intercept 36.32. A higher efficiency was achieved with four primers in PCDR in comparison to two primers used with either the PCDR master mix or TaqMan Master Mix.

Competing interests

G.F., C.H., and L.A. are employees of Oxitec Ltd; G.F. and L.A. have equity interest in Oxitec Ltd. Oxitec, and GeneFirst have patents or patent applications related to the subject matter of this paper. G.F. and L.A. have equity interest in GeneFirst Ltd., which is developing molecular diagnostics methods and products.

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