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PCR detection of nearly any dengue virus strain using a highly sensitive primer 'cocktail'

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PCR detection of viral pathogens is extremely useful, but suffers from the challenge of detecting the many variant strains of a given virus that arise over time. Here, we report the computational derivation and initial experimental testing of a combination of 10 PCR primers to be used in a single high-sensitivity mixed PCR reaction for the detection of dengue virus. Primer sequences were computed such that their probability of mispriming with human DNA is extremely low. A 'cocktail' of 10 primers was shown experimentally to be able to detect cDNA clones representing the four serotypes and dengue virus RNA spiked into total human whole blood RNA. Computationally, the primers are predicted to detect 95% of the 1688 dengue strains analyzed (with perfect primer match). Allowing up to one mismatch and one insertion per primer, the primer set detects 99% of strains. Primer sets from three previous studies have been compared with the present set of primers and their relative sensitivity for dengue virus is discussed. These results provide the formulation and demonstration of a mixed primer PCR reagent that may enable the detection of nearly any dengue strain irrespective of serotype, in a single PCR reaction, and illustrate an approach to the broad problem of detecting highly mutable RNA viruses.

Introduction

Molecular methods are of increasing importance in pathogen detection, and are gradually replacing serology and culturing in many applications. PCR is particularly widely used because of its great analytical sensitivity, but requires primers with perfect or close sequence match to the pathogen genome. Although it is often not difficult to design primers specific to an individual strain of a pathogen, genetic drift and selection produces a variety of sequence variants that can

be difficult to target effectively. This problem is especially pronounced with the mutation-prone RNA viruses.

Dengue virus is a rapidly emerging mosquito-borne positive-strand single-stranded RNA virus that infects an estimated 50 million people annually [1]. Dengue hemorrhagic fever is a severe form of dengue fever that claims approximately 12 500 reported lives every year. In the last four decades, dengue has spread from

Abbreviations

e-PCR, electronic-PCR; STS, sequence tagged site; NCBI, National Center for Biotechnology Information.

approximately 10 countries to 100 (World Health Organization: <http://www.who.int/mediacentre/factsheets/fs117/en/index.html>, accessed September 2010), transmitted by the mosquito vectors *Aedes aegypti* and *Aedes albopictus*. The virus occurs as four serotypes (DENV-1 to DENV-4); all four serotypes can co-circulate in affected areas.

Despite extensive ongoing efforts, no vaccines for dengue are yet available [2,3], and prevention can only be achieved by arresting the multiplication of mosquito vectors. For diagnosis, serological antigen-detection [4,5] and antibody-detection tests [6,7] and nucleic acid-based diagnostics [8–13] are in use, in addition to virus culture from infected samples. Antibody-detection serological tests depend upon the appearance of the host immune response 5–6 days after the onset of fever. Similarly, virus isolation from infected sera is a time-consuming process requiring 7 days of incubation followed by screening for the presence of virus [14]. Nucleic acid-based assays offer rapid and specific detection and serotyping of dengue virus, and are gradually replacing serological and culture techniques. These methods include nested RT-PCR [9], real-time RT-PCR [10,12,13], loop-mediated isothermal amplification [11], nucleic acid sequence-based amplification [15] and Taqman assays [8]. Although these methods are rapid, they are subject to false-negative results. As discussed below, the most widely cited early primer sets [9,10,13] can detect a significant fraction of dengue strains only through priming involving multiple mismatches, increasing the probability of false-negative results, induced by escape mutation or PCR failure.

Recently, we developed a set of novel algorithms [16] for exhaustive identification of all nucleotide subsequences present in a pathogen genome that differ by at least a chosen number of mismatches from the sequences of the host and/or other background genomes. Briefly, the algorithm scans the genome sequences of the target pathogen and the host, creating lists of all subsequences of a specified length n (n -mers) occurring in each genome. The subse-

quences present within the pathogen genome are then annotated according to the minimum number of base changes required to convert each subsequence to the nearest subsequence present in the host sequence. The pathogen subsequences furthest from the host genome are favored targets for probes or primers for the detection of that pathogen against that host background. It was found that 99.99% of all possible 11-mers, 70% of all 15-mers and 5% of all 18-mers are present in the human genome [16]. A select few ‘human-blind’ dengue primers have previously been described [17].

In this work, in addition to the distance from the nearest human sequence, primer sequences were also selected based on their melting temperature, absence of homopolynucleotide runs, predicted amplicon size and serotype specificity. Candidate dengue-specific, human-blind primers were further categorized according to the serotypes of the strains they were predicted to detect into five groups of primer pairs (Table 1 and Tables S1 and S2). Here, we report the preparation and testing of a mixture of 10, 18- to 22-nucleotide PCR primers, each of which is at least two mismatches away from the nearest human sequence. Following the nomenclature of Koekemoer *et al.* (2002) [18], we refer to this multiple primer pair/one template strategy as ‘cocktail PCR’ to differentiate it from multiplex PCR, in which more than one target is amplified. The cocktail is composed of one primer pair from each of the five primer pair groups, which together are predicted to detect nearly any strain of the four dengue virus serotypes. This cocktail is computationally predicted to detect 1610 of 1688 DENV strains listed in the Broad Institute Dengue Virus Database (<http://www.broad.mit.edu/annotation/viral/Dengue/Home.html>, accessed July 2009) as of July 2009, with perfect primer match, and 512 of 516 additional geographically dispersed strains obtained from National Center for Biotechnology Information (NCBI) in January 2011. Computational predictions of sensitivity* of the primer cocktail for the 2204 dengue strains considered and corresponding experiments with both dengue cDNA clones and

Table 1. Strain coverage of the five primer groups in set 1. Each entry is the number of the 163 design-basis strains in the row associated with that serotype covered by the primers of the group associated with that column; the primers are categorized according to the strains that they detect. The number of primer pairs in each group is indicated in the column header. See details in Table S1.

Dengue serotype (no. of strains in design basis set)	Number (percentage) of strains of row serotype covered by column primer group				
	Group 1 (1 primer pair)	Group 2 (1 primer pair)	Group 3 (48 primer pairs)	Group 4 (311 primer pairs)	Group 5 (35 primer pairs)
DENV-1 (38 strains)	7 (18.4%)	12 (31.5%)	37 (97.3%)	0	0
DENV-2 (64 strains)	60 (93.7%)	40 (62.5%)	0	0	0
DENV-3 (45 strains)	0	0	0	45 (100%)	0
DENV-4 (16 strains)	0	0	0	0	16 (100%)

viral RNA of all four serotypes are reported here. The results of this study demonstrate the use of these human-blind primers for specific dengue virus detection and their implementation in a primer cocktail strategy enabling high sensitivity for dengue strains and facilitating a rapid detection method. *In this paper, 'sensitivity' refers to the diagnostic sensitivity, which is different from analytical sensitivity. Diagnostic sensitivity is the indicator of true-positive calls for a pathogenesis, whereas analytical sensitivity is the detection limit of a detection method/assay [19].

Results

Human-blind dengue primers

Primers were tested for specific amplification of DENV cDNA in the presence of excess human DNA. The mass ratio of DENV to human DNA was 1 : 1000 and the molar ratio was 1 : 0.005 (a molar ratio of approximately 1 : 5 was also tested and showed identical results). Primers from set 1 were tested experimentally for optimum annealing temperature determination, human-blindness confirmation, single amplicon formation and cross-reactivity with other serotypes. Primers were also tested computationally to determine their strain sensitivity. The set 1, group 2 primer pair 1G2P1 was predicted to detect only 95% of DENV-2 strains, and it was replaced with a primer pair from set 2, group 2 (2G2P5) to increase predicted sensitivity for DENV-2 strains (to 100% of the strains tested).

Experimental testing found that the primers amplified dengue and not human DNA. As an example, amplification curves of DENV-4 (GU289913) with all five primer groups are shown in Fig. 1. As predicted (Table 1), no amplification of DENV-4 by primers from groups 2, 3 or 4 was observed, although group 1 primers showed an increase in fluorescence in the last (35th) PCR cycle. No amplification of human DNA was observed with any of the primers. Figure 2 shows agarose gel electrophoresis of the amplification products; identical amplicons were observed in the presence and absence of excess human DNA. The melting T_m of amplicons formed in the presence and absence of human DNA was 81.1 ± 0.28 °C ($n = 4$) and 81.1 ± 0.29 °C ($n = 10$), respectively. Furthermore, PCR was highly reproducible for each primer pair; coefficients of variation in C_t values for group 1 primer pair 1G1P1 (which detects both DENV-1 and DENV-2) were 2.0 and 0.9% for DENV-1 and DENV-2, respectively, and 1, 1.8, 4.4 and 3.4% for groups 2 (2G2P5), 3 (1G3P6), 4 (1G4P217) and 5 (1G5P30), respectively, with the strains that they detect.

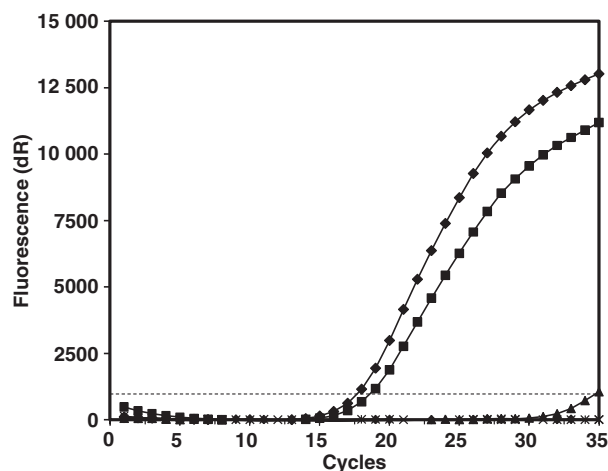


Fig. 1. Real-time PCR amplification of DENV-4 (GU289913) with and without human DNA. Group 5 primers (1G5P30, as used in the 'cocktail' mixture) amplified DENV-4 (GU289913) in the presence of 1000-fold excess human DNA (squares) and the absence of human DNA (diamonds) under optimal PCR conditions. Group 1 (triangles), group 2 (crosses), group 3 (circles) and group 4 (asterisks) primers showed inefficient or no amplification, as predicted. Group 1 primers showed some amplification in the last cycle of the PCR. The amplification threshold was set at a baseline-subtracted fluorescence value of 990 (horizontal line).

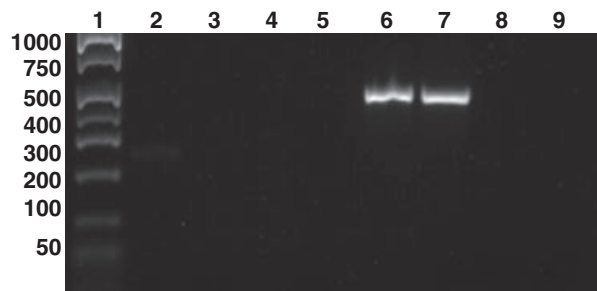


Fig. 2. Agarose gel electrophoresis of PCR products obtained with DENV-4 (GU289913). Lane 1, Hi-Lo DNA marker; lane 2, PCR with group 1 primers; lane 3, group 2 primers; lane 4, group 3 primers; lane 5, group 4 primers; lane 6, group 5 primers; lane 7, group 5 primers in the presence of 1000-fold excess human DNA; lane 8, group 5 primers with human DNA alone; lane 9, no-template control for group 5 primers. Each primer pair tested is a component of the highly sensitive primer cocktail discussed in this work (Table 2). PCR was performed at a consensus annealing temperature of 60 °C for 60 s, and extension at 72 °C for 90 s for 35 cycles.

Primers detected the serotypes they were predicted to detect; there were no false negatives for any of the primer groups. Specificity for dengue is expected to be very good; the primers were predicted to be specific to dengue virus when computationally tested against 291 strains of other nondengue flaviviruses, including strains of Japanese encephalitis virus, St. Louis encephalitis virus, West Nile virus and yellow fever virus (and also

Table 2. Primer pairs that make up the highly sensitive primer cocktail discussed in this work. Note that 1G1P1 is listed twice because it covers both DENV-1 and DENV-2, and that only the group 2 primer pair was taken from set 2. An average primer sequence location across multiple strains of each serotype is shown, together with the predicted average amplicon size. The primer orientation is 5' to 3'. Detailed information on the recognition of each of the 1688 Broad Institute database strains by each primer is given in Table S7.

Primer pair	Primer group	Primer sequence		Dengue serotype	Average amplicon location (nucleotides)	Average amplicon size (bp)
		5'-Forward-3'	5'-Reverse-3'			
1G1P1	Group 1	CAAACCATGGAAGCTGTACG	TTCTGTGCCTGGAATGATGCT	DENV-1	10451 10671	219
1G1P1	Group 1	CAAACCATGGAAGCTGTACG	TTCTGTGCCTGGAATGATGCT	DENV-2	10438 10660	221
2G2P5	Group 2	GAGTGGAGTGGGAAGGAGAAGGG	CCTCTTGGTGTGGTCTTTGC	DENV-2	9057 9305	248
1G3P6	Group 3	CAGACTAGTGGTTAGAGGAGA	GGAATGATGCTGTAGAGACA	DENV-1	10482 10661	179
1G4P217	Group 4	ATATGCTGAAACCGCTGAG	CATCATGAGACAGAGCGAT	DENV-3	104 382	279
1G5P30	Group 5	TTCCAACAAGCAGAACAACAT	GCTACAGGCAGCACGGTTT	DENV-4	9903 10318	415

against the genome of the carrier organism *Aedes aegypti*, which might be useful for insect screening). Specificity among DENV serotypes was very good, but not perfect; the DENV-3 cDNA clone was detected by primer groups beyond the expected (Table 1) group 4. As discussed below, these amplification products were predicted by electronic-PCR (e-PCR) when one mismatch and one gap were allowed. A very faint unpredicted amplification of DENV-4 by 1G1P1 primers (Fig. 2, lane 2 near 250 bp) was also observed in the last amplification cycle (Fig. 1). Amplification curves and the respective thermal dissociation curves of DENV-1, DENV-2 and DENV-3 cDNA with all five primer groups in the presence and absence of 1000-fold excess human DNA are shown in Figs S1–S6.

Primer testing with DENV and human RNA

Primers were further tested with total RNA extracted from DENV-1 (Piura, Peru)-, DENV-2 (New Guinea C)-, DENV-3 (Asuncion, Paraguay)- or DENV-4 (Dominica, West Indies)-infected C6/36 mosquito cells. Figure 3 shows a comparison of real-time amplification curves of total RNA extracted from DENV-2 (New Guinea C)-infected C6/36 cells (with and without RT), and uninfected C6/36 cells. As expected, only DENV-2-infected C6/36 cells showed amplification, and only in the presence of RT. No amplification was observed with total RNA of normal C6/36 *A. albopictus* cells or in the absence of template. DENV-2 was amplified by primers 1G1P1 and 2G2P5 and, as expected, not amplified by primers from groups 3, 4 and 5. Identical products were formed by PCR of DENV-2 cDNA and RT-PCR

of DENV-2 RNA with the 2G2P5 primer pair, as seen in the amplicon melting curves [Fig. 4; $T_m = 79.9 \pm 0.40$ °C ($n = 4$) and 79.7 ± 0.25 °C ($n = 4$), respectively] and by agarose gel electrophoresis (Fig. 5). Amplification and melting temperature curves of amplicons obtained by real-time RT-PCR with DENV-1, DENV-2, DENV-3 and DENV-4 RNA in the absence and presence of human whole blood total RNA are

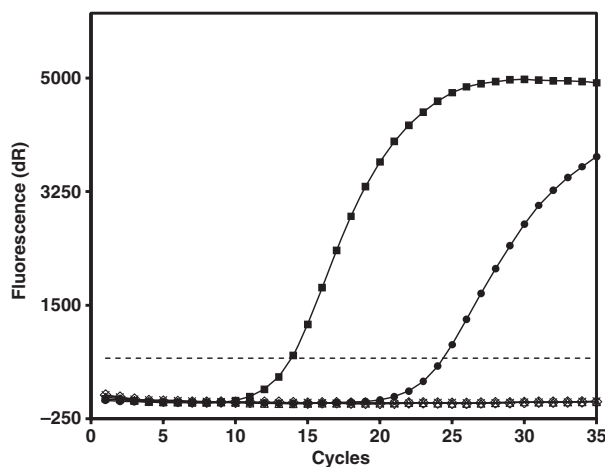


Fig. 3. Real-time RT-PCR of DENV-2 (New Guinea C)-infected C6/36 cell total RNA using cocktail primers (Table 2). Cocktail primers of group 1 (1G1P1), group 2 (2G2P5), group 3 (1G3P6), group 4 (1G4P217) and group 5 (1G5P30) were used. DENV-2 New Guinea C-infected C6/36 cell total RNA was amplified by group 1 (circles) and group 2 (squares) primers as predicted. No amplification was seen with any of the following: no-RT controls; uninfected C6/36 cell control; no-template control; and primers from any other of the three groups (not shown). The amplification threshold was set at a baseline-subtracted fluorescence value of 674 (horizontal line).

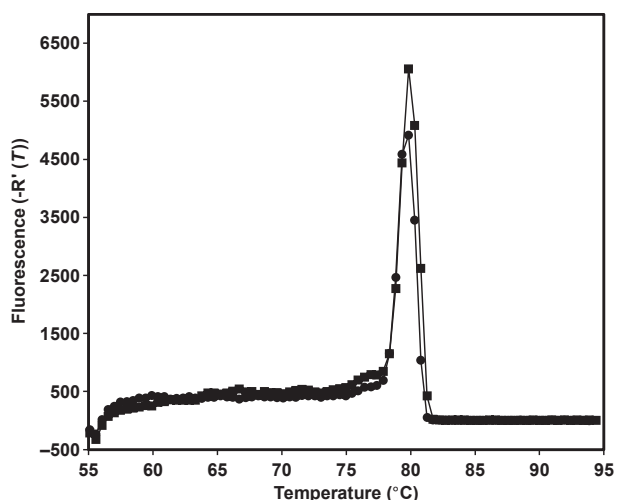


Fig. 4. Thermal dissociation curves of products of real-time RT-PCR amplification of DENV-2 New Guinea C (M29095) RNA (squares) and cDNA (circles). Total RNA of DENV-2 (New Guinea C)-infected C6/36 cells resulted in an identical PCR product with $T_m = 79.7^\circ\text{C}$, as compared with a DENV-2 cDNA clone with $T_m = 79.9^\circ\text{C}$. The results for group 2 primer pair 2G2P5 are shown.

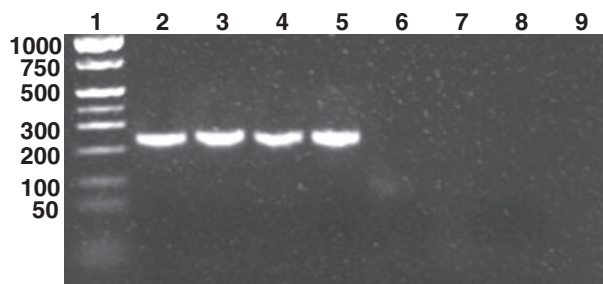


Fig. 5. Products of PCR amplification of DENV-2 New Guinea C (M29095) RNA and cDNA. Amplification of DENV-2 cDNA and total RNA from DENV-2 (New Guinea C)-infected C6/36 cells with the 2G2P5 primer pair in the absence and presence of human DNA or RNA. Lane 1, Hi-Lo DNA size marker; lane 2, PCR products obtained with 2G2P5 primers and DENV-2 cDNA plasmid clone; lane 3, DENV-2 (New Guinea C) cDNA in the presence of 1000-fold excess human genomic DNA; lane 4, DENV-2 (New Guinea C)-infected C6/36 cells total RNA; lane 5, DENV-2 RNA in the presence of 100-fold excess human whole blood total RNA; lane 6, human RNA alone; lane 7, uninfected C6/36 cells total RNA; lane 8, no-RT control of DENV-2-infected C6/36 cells total RNA; lane 9, no-template control. RT-PCR was carried out with 100 nM primer concentration at 60°C annealing temperature, for 35 cycles.

shown in Figs S7–S14. Identical amplicons were obtained in the absence and presence of a 100-fold mass excess of human RNA. Primer pair 2G2P5 gave weak amplification very late in the PCR (at cycle 33–35) with

human blood total RNA; the amplification was too weak for the product to be observable in Fig. 5.

Cocktail PCR

A major goal of this work was to advance the development of a single-PCR diagnostic tool with broad sensitivity across dengue strains and serotypes. In support of this goal, after validating the sensitivity and specificity of the individual primer pairs, we blended five primer pairs together to produce a ‘cocktail’ expected to give one or more products with any of the dengue virus strains used in the primer design, representing all four serotypes. In contrast to multiplex PCR, in this assay, multiple products are not essential (or problematic), but could potentially contribute additional information upon electrophoretic analysis and might increase the sensitivity to strains not considered in the design.

Experimentally, a single cocktail of primers was found to be able to detect test strains representing all of the DENV serotypes. All serotypes, with the exception of DENV-4, produced expected multiple amplicons with the 10-primer cocktail, as seen by electrophoretic analysis (Fig. 6A). The amplicon band pattern observed was not affected by the presence of excess human DNA. No template and human DNA controls did not show any amplification. Amplicons obtained with real-time RT cocktail PCR of all four serotypes of DENV RNA in the absence and presence of human RNA (Fig. 6B) were not affected by the presence of excess human RNA. No template (not shown) and human RNA-only controls showed no amplification.

Multiple amplicons were obtained with a single template, as expected in cocktail PCR. Products obtained by the amplification of a sequence lying between the sites recognized by a forward primer belonging to one group and a reverse primer belonging to another group were termed ‘hybrid’ products. The multiple hybrid products generated from most templates (see Fig. 6A) were observed to be predictable (see Table S3) and highly reproducible, and could potentially be used to identify serotypes or even genotypes. The existence of multiple amplicons may enhance resistance to false negatives produced by escape mutants of these mutable RNA viruses.

e-PCR-based dengue virus detection sensitivity

Amplification by the primer cocktail was predicted by e-PCR for all 1688 strains in the Broad Institute Dengue Virus Database as of July 2009, and by

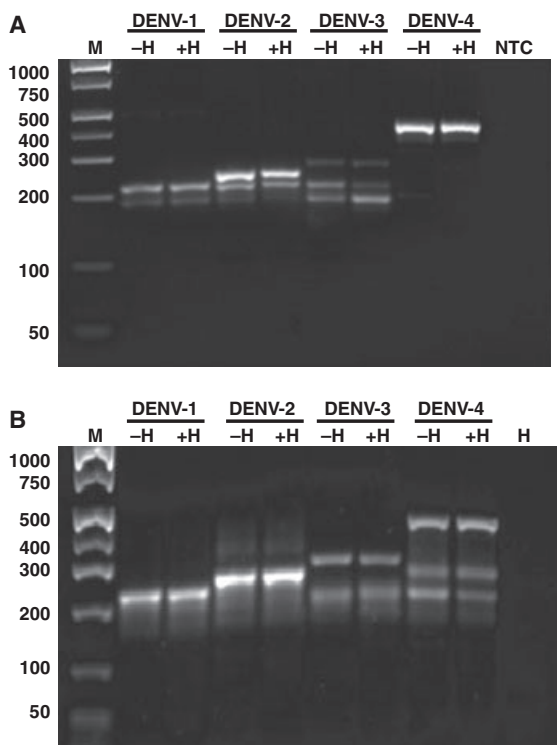


Fig. 6. Ten-primer cocktail PCR with all four serotypes: band patterning. Amplification with 10 primers (25 combinations) gives multiple specific products, as predicted (see Table S3). PCR products of amplification of dengue cDNA clones (A) and RNA (B) (DENV-1, DENV-2, DENV-3 and DENV-4) using primer cocktail in the absence (-H) and presence (+H) of human DNA where the mass ratio of human DNA to dengue cDNA was 1000 and human RNA to dengue RNA was 100–1000, as visualized by agarose gel electrophoresis. M, Hi-Lo DNA size marker; NTC, no-template control; H, human RNA alone.

MegaBLAST for 516 additional geographically dispersed strains obtained from NCBI in January 2011. Of the 1688 available dengue virus genome sequences, the primer cocktail was predicted by e-PCR to detect 1610 (95%), with perfect primer matches (Table 4; Table S4), missing 3.4% of 748 DENV-1, 0.5% of 568 DENV-2, 14.5% of 316 DENV-3 and 5.3% of 56 DENV-4 strains tested. With reduced stringency, allowing one mismatch and one insertion per primer, 1675 of 1688 strains were predicted to be detected (99%) (Table 4; Table S5). Of the 13 ‘missed’ strains, the two belonging to the DENV-2 serotype (accession numbers FJ913016.1 and GQ199605.1) were partial genome sequences missing both primer target regions. The remaining 11 strains (all DENV-1) were analyzed for sequence match with both primer groups 1 and 3 using BLASTN, as primers from both groups can detect DENV-1. The group 1 primer pair 1G1P1 did not

show a significant match to any of these 11 strains (the group 1 forward primer had four mismatches with all strains and the reverse primer had seven to 13 mismatches). The group 3 primer pair 1G3P6F forward primer matched eight strains perfectly, two strains (FJ850075 and FJ850073) with one mismatch and missed one strain (FJ639812) with four mismatches. The reverse primer, 1G3P6R showed three to 13 mismatches with these 11 strains.

Geographical variation in dengue virus

Of the 516 strains with complete genome sequences analyzed, 512 were predicted to be detected by the primer cocktail (Table S6). The four missed strains belonged to DENV-1 (GenBank accession numbers FJ469907, FJ469908, FJ469909 and HM181969). As before, group 1 primers did not show any match to these missed strains and group 3 forward primers matched perfectly. The group 3 reverse primer showed no match. These sequences were found to be 10 454–10 642 bp long. As the average primer location of group 3 primers is $10\,661 \pm 4.8$ bp, based on 729 DENV-1 genome sequences (Table S7), it is likely that these strains were predicted not to be detected because of a missing sequence at the 3’ end.

Amplification efficiencies and analytical sensitivity of the primers

Amplification efficiencies calculated for primer pair-optimized PCR conditions and consensus cocktail PCR conditions are shown in Table 3. The consensus reaction conditions represent a workable compromise for all the primers in a single-tube reaction. Cocktail amplification efficiencies, therefore, are not identical to those under conditions optimized for a single primer pair. Under optimal PCR conditions, at the amplification efficiencies values listed in Table 3, the detection limit of the dengue cDNA plasmid clones was 2.5 molecules· μL^{-1} for all serotypes and primer groups, with the exception of group 5 primers with DENV-4 where the detection limit was 25 molecules· μL^{-1} . Under compromise consensus cocktail PCR conditions, at the amplification efficiency values listed in Table 3, the detection limit of the dengue cDNA plasmid clones was 2400 molecules· μL^{-1} for DENV-1, 24 molecules· μL^{-1} for DENV-2, 240 molecules· μL^{-1} for DENV-3 and 24 000 molecules· μL^{-1} for DENV-4. The detection limit for DENV-4 improved 100-fold to 240 molecules· μL^{-1} when the concentration of group 5 primer pair 1G5P30 was doubled to 100 nM in the primer cocktail.

Table 3. Amplification efficiency of primer pairs. Efficiency is reported under conditions optimized for each primer pair and also for the consensus cocktail PCR conditions: primer concentration 50 nM each; annealing temperature 60 °C; extension time 60 s ($n \geq 3$; $R^2 \geq 0.995$; average $R^2 = 0.997$).

Primer pair	Primer sequence		Template	Average efficiency \pm SD	
	5'-Forward-3'	5'-Reverse-3'		Optimal PCR conditions	Cocktail PCR conditions
1G1P1	CAAACCATGGAAGCTGTACG	TTCTGTGCCTGGAATGATGCT	DENV-1	90.0 \pm 1.50	89.5 \pm 2.52
1G1P1	CAAACCATGGAAGCTGTACG	TTCTGTGCCTGGAATGATGCT	DENV-2	98.9 \pm 6.23	98.9 \pm 6.23
2G2P5	GAGTGGAGTGAAGGAGAAGGG	CCTCTTGGTGTGGTCTTTGC	DENV-2	98.4 \pm 0.84	98.4 \pm 0.84
1G3P6	CAGACTAGTGGTTAGAGGAGA	GGAATGATGCTGTAGAGACA	DENV-1	92.1 \pm 0.57	73.6 \pm 2.31
1G4P217	ATATGCTGAAACGCGTGAG	CATCATGAGACAGACGAT	DENV-3	90.3 \pm 1.09	83.9 \pm 5.16
1G5P30	TTCCAACAAGCAGAACAACAT	GCTACAGGCAGCACGGTTT	DENV-4	81.8 \pm 5.03	79.0 \pm 1.83

Table 4. Comparison of dengue strain coverage with primers of previously published studies using e-PCR. e-PCR predictions were performed with the requirement of perfect match (first number in each cell) and also allowing up to one mismatch and one gap per primer (second number in each cell).

Dengue serotypes	Number of strains tested	Present study	Lai <i>et al.</i> [13]	Lanciotti <i>et al.</i> [9]	Lo <i>et al.</i> [10]
DENV-1	748	722/737	0/619	0/0	623/737
DENV-2	568	565/566	152/456	0/0	219/532
DENV-3	316	270/316	0/307	0/251	309/316
DENV-4	56	53/56	0/55	0/1	0/54
TOTAL	1688	1610/1675	152/1437	0/252	1151/1639

Discussion

We describe the formulation of a universal primer reagent predicted to detect 1610 of the 1688 dengue strains, irrespective of serotype, curated in the Broad Institute Dengue Virus Database, as of July 2009. We demonstrated the broad strain sensitivity of this reagent using DENV cDNA clones and RNA of the four serotypes in the presence of a vast excess of human DNA and RNA. The reagent has high analytical sensitivity and specificity to the presence of dengue virus cDNA clones, even in a vast excess of contaminating human DNA. Serotyping potentially could be achieved by electrophoretic analysis of hybrid products (Fig. 6A, B; all predicted products of amplification of each of the 1688 tested strains with the 10 cocktail primers and with the primers of Lanciotti *et al.* [9], Lo *et al.* [10] and Lai *et al.* [13] are tabulated in Tables S4 and S5). Serotyping could also be achieved by using these primer pairs in separate reactions. However, our

immediate goal was high sensitivity for a broad range of dengue virus strains.

The predicted strain sensitivity of PCR using the cocktail described in this work was compared using e-PCR with the predicted sensitivities of some earlier, widely cited primers [9,10,13]. To maintain uniformity, the multiple primers of previous studies were also treated as a cocktail and, hence, detection by any possible hybrid pairs was also considered (Table 4; details in Tables S4 and S5). It should be noted that previously described primers, particularly those of the pioneering and widely cited study of Lanciotti *et al.* [9] have lost some predicted sensitivity with the sequencing of a very large number of additional dengue strains since that time.

On lowering the stringency of search by allowing one mismatch and one gap per primer, the sensitivity of all primer sets increased such that of the 1688 dengue strains, the present primer cocktail was predicted to detect 1675 strains, whereas the previous primer sets

were predicted to detect 1437 [13], 252 [9] and 1639 [10] strains, as shown in Table 4. It should be noted that multiple mismatch priming can enhance empirically observed sensitivity beyond that predicted computationally. Although this primer cocktail is predicted to have excellent sensitivity, it fails to detect ~ 5% of the strains and further analysis is required to determine the causes. Sequence variation due to wide geographical distribution did not have an effect on the performance of the cocktail, supporting the potential use of these primers globally. The primer cocktail reagent will enable sensitive and specific dengue virus detection when serotyping is not immediately required. Such a method will be helpful to a dengue-specific drug therapy, when it is available [20]. Rapid detection is also of importance to prevent the development of dengue hemorrhagic fever. Additionally, the broad sensitivity of the primer cocktail will also aid in better epidemiological characterization of the virus.

These results provide a demonstration of the high projected sensitivity of human-blind dengue primers and the operation of the primer cocktail strategy for dengue virus detection. These primers are available for testing with dengue strains at a larger scale to support the development of a rapid clinical PCR detection method. Perhaps most importantly, the methodology described in this work could be generally applied to the problem of developing broadly useful diagnostics for mutable pathogens, especially RNA viruses.

Materials and methods

Primer selection

Potential primers (18–22 nucleotides in length) derived from an exhaustive search of 163 dengue virus genomes were screened against the complete human genome (build 34). Primers that differed from the nearest sequence in the human genome by at least two mismatches were subjected to further screening using PCR primer design criteria [16]. The expected melting temperature T_m was calculated using the nearest-neighbor model of SantaLucia *et al.* [21] and was required to be between 50 and 65 °C; homopolynucleotide stretches of more than three bases were not allowed. Primers that passed this screening were paired based on T_m difference; expected amplicon size with dengue templates and potential for primer–dimer formation. The melting temperatures of the forward and reverse primer of each pair were required to differ by < 5 °C and the predicted amplicon length was required to be 150–500 bp. Primer pairs were rejected on the basis of possible primer–dimer formation if a candidate primer pair had four or more consecutive complementary nucleotides.

Primer strain coverage and serotype specificity

A set of human-blind candidate primer pairs was identified using all 163 dengue virus genome sequences recorded in GenBank as of March 2007, and termed set 1 (Table 1 and Table S1). Five groups of primer pairs emerged when these were categorized based on the strain coverage of each primer pair. Notably, in set 1, grouping the primers by dengue strain coverage resulted in five groups, two of which (groups 1 and 2) consisted of only one primer pair each. Against the possibility that one or both of these single primer pairs would fail to meet selection criteria and/or experimental validation, another choice of primers for group 1 and group 2 was selected and was referred to as set 2 (Table S2). Set 1 consisted of 396 primer pairs categorized into five groups according to their strain coverage (Table 1). For instance, any one of the 48 primer pairs in group 3 is predicted to detect 37 of the 38 DENV-1 strains in the 163-strain design basis set, and no strain of any other serotype. By selecting one primer pair from each group, nearly any of the 163 design-basis dengue strains may in principle be detected. Thus, a cocktail combining one primer pair from each of the five groups from set 1 or set 2 is predicted to be able to detect almost any of the 163 strains, covering all four serotypes.

Flavivirus specificity of dengue primers

The specificity of the dengue primers was tested against 291 nondengue flaviviruses, including 67 strains of Japanese encephalitis virus, 28 strains of St Louis encephalitis virus, 172 strains of West Nile virus and 24 strains of yellow fever virus using BLASTn [22]. The genome sequences of the flaviviruses were retrieved from Flavitrack (<http://carnot.utmb.edu/flavitrack/>; [23,24]). The primers were also tested against the genome of the carrier mosquito *Aedes aegypti*.

Primers tested in the present study

For the present study, one primer pair was selected from each of the five groups, originally from set 1. This was done by first testing the single primer pair in group 1 and group 2 and 10 randomly selected primer pairs each from groups 3, 4 and 5 from set 1. Subsequent selection was based on empirical performance under standard PCR test conditions of 100 nM primer concentration and 60 °C annealing temperature. These conditions were considered desirable for amplification under cocktail PCR conditions where multiple primers are required to be functional and the annealing temperature needs to be stringent. The chosen primers (Table 2) were empirically tested for sensitivity, specificity and amplification efficiency with one strain of each serotype and then subjected to computational testing against all 1688 dengue strains in the Broad Institute Dengue Virus Database, as of July 2009. The sensitivity of the set 1,

group 2 primers for multiple strains was found to be low, and they were replaced with set 2, group 2 primers. The source of the primer pair is represented in the nomenclature used below. For example, the primer name 2G2P5 identifies that the primer pair is from set 2, group 2, and is primer pair number 5 in serial order within that group (see listings in Tables S1 and S2).

Dengue virus templates for experimental testing

Primers were initially tested with dengue virus cDNAs cloned in the yeast–*Escherichia coli* shuttle vector pRS424. DENV-1 West Pacific (U88535), DENV-2 New Guinea C (M29095), DENV-3 (FJ639719) and DENV-4 (GU289913) clones were kindly provided by B. Falgout, B. Zhao and R. Levis of the US Food and Drug Administration.

Following tests with cDNA clones, primers were also tested with DENV-1 (Piura, Peru), DENV-2 New Guinea C (M29095), DENV-3 (Asuncion, Paraguay) and DENV-4 (Dominica, West Indies) RNA. The DENV-infected C6/36 mosquito (*A. albopictus*) cells and uninfected C6/36 mosquito cell samples were generously provided by R. B. Tesh, Director of the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch at Galveston, TX, USA. Samples were supplied as TRIzol™ (Invitrogen, Carlsbad, CA, USA) extracts and further purified by phenol/chloroform extraction to obtain total RNA from both infected and control normal cells. Total RNA was used as the template for real-time RT-PCR. Dengue virus cDNA and RNA were tested in the absence or presence of a large excess of human genomic DNA (1000-fold by mass) or human whole blood RNA (100–1000-fold by mass) extracted using QIAamp Blood RNA Mini kit (Qiagen, Valencia, CA, USA) to demonstrate the human RNA-blind property of the primers. Anonymized normal donor blood was purchased from Gulf Coast Regional Blood Center (Houston, TX, USA).

PCR amplification of dengue cDNA clones and cocktail PCR

PCR reactions were conducted in 25 µL containing up to 100 pg (~ 6 million plasmid copies, or in dilution series for efficiency determinations as described below) of cDNA template (added in 1.0 µL), 12.5 µL 2 × Brilliant® II SYBR® Green Q-PCR master mix, 100 nM of each forward and reverse primer and nuclease-free water. Identical thermocycling conditions were used for all five groups of primers – initial activation of polymerase (95 °C, 10 min), followed by 35 cycles of DNA denaturation (95 °C, 1 min), primer annealing (60 °C, 1 min) and primer extension (72 °C, 40 s). Controls omitting DNA template were included in each experiment. An Mx3005P™ QPCR system (Agilent Technologies, Santa Clara, CA, USA) was used for thermo-

cycling and its software MXPRO version 3.04b was used for data collection and analyses. The coefficient of variation in the C_t values was obtained by dividing the standard deviation by the arithmetic mean of the amplification C_t values. All experiments were carried out in triplicate on different days. Amplicons were visualized after 1.5% agarose gel electrophoresis using SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA).

To produce the broad-sensitivity primer cocktail, one primer pair each from the five primer groups was mixed together such that the final concentration of each primer in the PCR reaction was 50 nM. Other PCR conditions were identical to those described above except that the extension time was increased to 60 s.

Real-time RT-PCR amplification of dengue RNA and cocktail RT-PCR

Real-time RT-PCR was employed to detect DENV-1 (Piura, Peru), DENV-2 (New Guinea C), DENV-3 (Asuncion, Paraguay) or DENV-4 (Dominica, West Indies) RNA present in total RNA extracted from infected C6/36 mosquito cells. cDNA was synthesized directly using the primers described in Table 2. In a 25 µL PCR reaction, either 100 pg of DENV-1-, 1 ng of DENV-2-, 100 pg of DENV-3- or 1 ng of DENV-4-infected C6/36 cells total RNA was used for cocktail PCR. The primer cocktail was composed of 100 nM each of 1G1P1, 1G3P6, 1G4P217, 1G5P30 and 50 nM of 2G2P5 primer pairs (Table 2). PCR was carried out in the absence or presence of human RNA. All tests with spiked human RNA were performed unblinded. Brilliant® II SYBR® Green QRT-PCR master mix kit, 1-Step (Agilent Technologies) and Mx3005P™ QPCR system were used for thermocycling. MX3005P software version 3.04b was used for data collection and analyses, with the ‘amplification-based threshold’ algorithm and an adaptive-baseline correction used to determine the threshold cycle C_t . The amplification was considered positive when the C_t value was < 30 cycles. The mean T_m of the amplicons with standard deviation or T_m curves were reported when comparing the amplification of cDNA and RNA, or amplification in the absence and presence of human nucleic acids.

Primer amplification efficiencies

Standard curves were constructed by amplification of a 10-fold dilution series of each of the four dengue cDNAs with their respective primer pairs. Template amounts of 1 fg (~ 60 cDNA copies) to 10 ng (600 million cDNA copies) were used, together with no-template controls. The primer concentration and annealing temperature were optimized for each primer pair (Table S8), and each was also tested under the consensus ‘cocktail’ conditions. The extension time was adjusted in the range of 40–90 s, depending on the length of the expected amplicon (180–415 nucleo-

tides). An extension time of 60 s was used for consensus cocktail PCR; see above.

Amplification efficiency of primers under consensus cocktail PCR conditions

The amplification efficiency of each primer pair also was determined under the consensus cocktail PCR conditions (50 nM each primer, annealing temperature 60 °C, extension time 60 s). A seven point standard curve was constructed by amplification of a dilution series (template amount 1 fg to 1 ng) of each of the four dengue cDNAs with their respective primer pairs (Table 2). Nontemplate controls were included. Agilent's MXP software v3.04b uses a least mean squares curve fitting algorithm to generate standard curves by plotting the initial template amount on the x-axis and the threshold cycle (C_t) on the y-axis. The PCR efficiency is given by $10^{(-1/\text{slope})} - 1$, where the slope is -3.322 when the efficiency is 100% [25]. The R^2 value is also reported.

Reverse e-PCR testing of the primer cocktail with 1688 strains of dengue virus

To predict the sensitivity of the primer cocktail to diverse strains of the virus, reverse e-PCR [26] was employed to search viral sequences with the candidate primer pairs as query sequences for sequence tagged sites (STSs). In this calculation, an STS is defined by a primer pair flanking the site in appropriate orientation and the length of the STS is the expected PCR product size. The five forward and five reverse primers of the cocktail were considered in all 25 possible forward-reverse pairings (Table S3). Dengue virus genome sequences were obtained and downloaded from the Broad Institute Dengue Virus Database so that the reverse e-PCR could be run locally. Search parameters included either a perfect match between the primer and the dengue sequence or a maximum of one mismatch and one gap allowed per primer; the expected PCR product size was required to be 50–1000 bp. Additionally, the sensitivity of previously published primer sets (as cocktails) was predicted for comparison.

Effect of geographical variation in dengue virus on the performance of the primer cocktail

Each of the four serotypes of dengue virus can be classified into several genotypes, defined as a group of viruses having no more than 6% sequence divergence [27]. To predict the performance of the primer cocktail when tested with geographically widespread dengue strains, 516 strains of DENV-1, DENV-2 and DENV-3 were analyzed. DENV-4 was not considered in detail in this analysis because only 87 DENV-4 strains with complete genome sequences are recorded in the NCBI GenBank database (accessed January 2011), and all 87 strains were predicted to be detected by the primer cocktail (using MegaBLAST

[22]), specifically by the primer pair 1G5P30 with perfect primer match.

The genotype classification of each strain was determined either by referring to the published literature [28–34] or by using the Dengue Genotype Determination Tool of the Viral Bioinformatics Research Center (<http://denguedb.org>), which uses PAUP to generate the phylogenetic tree location for the query sequence. Of the 516 strains analyzed, 140 belong to DENV-1, 228 to DENV-2 and 148 to DENV-3. The genotype and source country of each strain analyzed are provided in Table S6. Strain geographical distribution information was obtained from NCBI GenBank and the NIAID Virus Pathogen Database and Analysis Resource (ViPR) online (<http://www.viprbrc.org>).

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Supporting information

The following supplementary material is available:

Fig. S1. Real-time PCR amplification curve of DENV-1 West Pacific (U88535) cDNA in the absence and presence of 1000-fold excess human DNA.

Fig. S2. Melting temperature curve of DENV-1 cDNA amplicons in the absence and presence of 1000-fold human DNA.

Fig. S3. Real-time PCR amplification curve of DENV-2 New Guinea C (M29095) cDNA in the absence and presence of 1000-fold excess human DNA.

Fig. S4. Melting temperature curve of DENV-2 New Guinea C (M29095) cDNA amplicons in the absence and presence of 1000-fold excess human DNA.

Fig. S5. Real-time PCR amplification curve of DENV-3 (FJ639719) cDNA in the presence and absence of 1000-fold excess human DNA.

Fig. S6. Melting temperature curve of DENV-3 cDNA amplicons in the absence and presence of 1000-fold human DNA.

Fig. S7. Real-time PCR amplification curve of DENV-1 (Piura, Peru) RNA in the absence and presence of 100-fold excess human whole blood total RNA.

Fig. S8. Melting temperature curve of DENV-1 RNA amplicons in the absence and presence of 100-fold human RNA.

Fig. S9. Real-time PCR amplification curve of DENV-2 New Guinea C (M29095) RNA in the absence and presence of 100-fold excess human whole blood total RNA.

Fig. S10. Melting temperature curve of DENV-2 RNA amplicons in the absence and presence of 100-fold human RNA.

Fig. S11. Real-time PCR amplification curve of DENV-3 (Asuncion, Paraguay) RNA in the absence and presence of 100-fold excess human whole blood total RNA.

Fig. S12. Melting temperature curve of DENV-3 RNA amplicons in the absence and presence of 100-fold human RNA.

Fig. S13. Real-time PCR amplification curve of DENV-4 (Dominica, West Indies) RNA in the absence and presence of 100-fold excess human whole blood total RNA.

Fig. S14. Melting temperature curve of DENV-4 RNA amplicons in the absence and presence of 100-fold human RNA.

Table S1. Human-blind dengue primers set 1.

Table S2. Human-blind dengue primers set 2.

Table S3. Hybrid amplicons – predicted and observed.

Table S4. e-PCR predicted results with primers from the present work, Lai *et al.* [13], Lanciotti *et al.* [9] and Lo *et al.* [10] with all 1688 dengue genome sequences retrieved from the Broad Dengue Virus Database, allowing no mismatches and no gaps.

Table S5. e-PCR predicted results with primers from the present work, Lai *et al.* [13], Lanciotti *et al.* [9] and Lo *et al.* [10] with all 1688 dengue genome sequences retrieved from the Broad Dengue Virus Database, allowing up to one mismatch and one gap.

Table S6. Dengue genotypes predicted to be detected.

Table S7. Amplicon locations of primers used in the study.

Table S8. Reaction conditions giving the highest PCR amplification efficiency for each individual primer pair used in the ‘cocktail’.

This supplementary material can be found in the online version of this article.

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