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Anchored Pan Dengue RT-PCR and Fast Sanger Sequencing for Detection of Dengue RNA in Human Serum

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10 4 **Running head:**
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12 5 Pan Dengue RT-PCR and Fast Sanger Sequencing
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18 7 Zhe Hu^{1,2,3}, Henrik Nordström^{2,4*}, Norbert Nowotny⁵, Kerstin I. Falk^{2,4}, and Gunnar
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20 8 Sandström^{2,6}
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27 10 ¹Key Laboratory of Zoonosis, Jilin University, Changchun, China;
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29 11 ²Swedish Institute for Infectious Disease Control, Solna, Sweden;
30

31 12 ³State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute,
32
33 Chinese Academy of Agricultural Sciences, Harbin, China;
34

35 13
36 14 ⁴Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm,
37
38 Sweden;
39

40 15
41 16 ⁵Clinical Virology, Department of Pathobiology, University of Veterinary Medicine, Vienna,
42
43 Austria;
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45 17
46 18 ⁶Karolinska Institutet, Department of Laboratory Medicine, Division of Clinical
47
48 Microbiology, Karolinska University Hospital, Huddinge, Stockholm, Sweden.
49

50 20
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54 21 ***Correspondence to:**
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56
57 22 Henrik Nordström
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59
60 23 Swedish Institute for Infectious Disease Control, Nobels väg 18, SE-17182 Solna, Sweden.

1
2
3 1 Phone: +46 (0)8 4572581.
4

5
6 2 Fax: +46 (0)8 307957.
7

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9 3 E-mail: henrik.nordstrom@smi.se.
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17 6 Dengue virus, 3' genome end, viral RNA, human serum, Pan Dengue RT-PCR, Fast Sanger
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19 7 Sequencing
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For Peer Review

1 Abstract

2 A large number of human infections are caused by different dengue virus strains, mainly in
3 the tropical and subtropical parts of the world, but also outside the endemic regions. RT-PCR
4 methods are used widely for detection of dengue virus RNA in acute-phase serum samples;
5 however, new sequence variation can inhibit these methods.

6 An assay was developed integrating an anchored Pan Dengue RT-PCR with a new Fast
7 Sanger sequencing protocol. For broad detection and identification of dengue virus RNA,
8 including new strains of all serotypes, the conserved 3' genome end was targeted for highly
9 specific cDNA synthesis. A combination of degenerated primers was used for second strand
10 synthesis, followed by tag primed amplification. The mixture of generated amplicons was
11 identified directly by the Fast Sanger sequencing from the anchored 3' genome end.

12 Evaluating the assay on human serum RNA spiked with viral RNA representing the four
13 dengue serotypes demonstrated a detection limit of 44-124 copies viral RNA per reaction for
14 a two-step format of the anchored Pan Dengue RT-PCR and 100-500 copies for a one-step
15 protocol, respectively. The different serotypes were clearly identified from the generated
16 sequences. Further, the 5-hour procedure was evaluated and compared to standard real-time
17 RT-PCR protocols on acute-phase serum samples from patients with confirmed dengue
18 infections.

19 This assay demonstrates a strategy for virus detection, which combines nucleic acid
20 amplification adapted for dengue virus RNA with direct and rapid sequencing. It provides a
21 tolerance for new sequence variation and the strategy should be applicable for other RNA
22 viruses.

1 Introduction

2 Dengue viruses cause the most prevalent arbovirus disease in tropical and subtropical regions
3 of the world, and a significant number of cases of imported disease outside the endemic
4 regions. There are four serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4),
5 all of which can cause dengue fever (DF) as well as the more serious dengue hemorrhagic
6 fever (DHF) or dengue shock syndrome (DSS). About 50 million cases occur throughout the
7 world each year [Guzman and Kouri, 2002], with 500,000 people affected by DHF or DSS
8 [Gubler, 1998; World Health Organization, 2004; World Health Organization, 2009]. Clinical
9 diagnosis of dengue virus infections is often difficult due to the wide range of clinical
10 symptoms. The NS1 antigen assays provide a rapid and simple detection of virus early in the
11 course of disease but without differentiation between the serotypes [McBride, 2009]. In
12 contrast, a number of rapid and serotype specific real-time RT-PCR assays for detection of
13 dengue virus RNA in acute phase samples have been reported [Kong et al., 2006; Lai et al.,
14 2007; Singh et al., 2006]. In travelers with acute infection outside the endemic regions with
15 limited information about potential strains, pan dengue real-time RT-PCR assays that can
16 detect all four serotypes in one reaction, are useful [Leparc-Goffart et al., 2009]. However, a
17 major limitation of all types of real-time RT-PCR assays is the considerable nucleic acid
18 sequence variation that many RNA viruses display, which may inhibit amplification by
19 preventing binding of primers, and thus produce a false-negative result. Another limitation is
20 the risk of non-specific amplification which can be reduced by inclusion of probes, such as
21 TaqMan, to increase the specificity. But the possibility of unknown sequence variation in both
22 the primer and probe binding sites makes the assays even more vulnerable to false-negative
23 results. Therefore, subsequent verification by Sanger sequencing of the generated amplicons
24 is used widely instead of including probes. In comparison, Sanger sequencing also produces a
25 more informative result. The faster Sanger sequencing protocol developed recently is both

1 work and time-saving, taking around a quarter of the time of the standard protocol. The
2 protocol is easy to incorporate into a routine procedure instead of using it as an additional
3 confirmation, and allows for development of new types of nucleic acid based assays. By
4 omitting the conventional identification of specific amplification products by size,
5 amplification curve or melting temperature, the RT-PCR amplification can be designed for an
6 improved detection of viral RNA.

7 In the present study an assay was developed for detection and sequence identification of
8 dengue virus RNA by combining a Pan Dengue RT-PCR with the Fast Sanger sequencing
9 (Fig. 1).

10 The short time and little effort required for the Fast Sanger sequencing protocol made it
11 feasible to incorporate into the assay procedure. Thereby it was possible to design an
12 anchored Pan Dengue RT-PCR amplification that took advantage of the direct sequencing to
13 achieve also the potential for the detection of new dengue virus strains of all serotypes in one
14 reaction. Primarily it relies on one specific primer that targets a highly conserved sequence
15 motif for cDNA synthesis, to anchor the amplification and the subsequent Fast Sanger
16 sequencing. The motif consisted of a conserved sequence at the outermost 3' genome end,
17 with potential involvement in secondary structures with biological importance [Shurtleff et al.,
18 2001]. A combination of degenerated primers was used for second strand synthesis, providing
19 limited dengue virus specificity and thereby some tolerance for new sequence variation. The
20 following amplification targeted a mutual tag primer on the 5' end of the primers used for
21 cDNA and second strand synthesis, both to facilitate the application of highly degenerated
22 second strand primers as well as to find the optimal amplification conditions for a
23 combination of several primers. Fast Sanger sequencing was carried out on the mixture of
24 generated amplicons from the anchored 3' genome end using the cDNA primer. Both one-

- 1 step and two-step formats of the Pan Dengue RT-PCR were tested for the evaluation of the
- 2 assay using serum RNA spiked with viral RNA as well as on clinical samples.

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1 **Materials and Methods**

2 **Virus strains and clinical samples**

3 DENV-1 strain Hawaii (GenBank accession no EU848545), DENV-2 New Guinea C
4 (AF038403), DENV-3 H87 (M93130) and DENV-4 H241 (AY947539) were cultured on Vero
5 cells (ATCC CCL-81) in a BSL-3 facility at the Swedish Institute for Infectious Disease
6 Control. Human serum for preparation of spiked samples was obtained from the Department
7 of Clinical Immunology and Transfusion Medicine, Karolinska University Laboratory,
8 Huddinge. Serum samples confirmed to contain virus RNA in routine diagnosis of dengue
9 (the procedures were described previously [Lindegren et al., 2005]), with multiplex endpoint
10 RT-PCR and real-time RT-PCR, were obtained at the Swedish Institute for Infectious Disease
11 Control. This study only includes confirmed samples without providing any additional
12 clinical information. According to a decision by the Stockholm Regional Ethical Review
13 Board, ethical approval is not needed for this type of study.

15 **Primer design**

16 For primer design all sequences available at NCBI GenBank (as of September 2008)
17 representing the four dengue serotypes were assembled and aligned in a step-wise procedure
18 to create minority consensus sequences for each serotype. The minority consensus sequences
19 were degenerated to include all found sequence variations in each position. A Perl script was
20 written to separate sequences from different serotypes into FASTA files, by local Stand-alone
21 Blast search (NCBI) against a database with representative full genome sequences of each
22 serotype. From each file the most similar sequences were assembled in SeqMan II (Dnastar,
23 Inc., Madison, WI, USA) to contigs from which minority consensus sequences were created.
24 These sequences were then aligned in MegAlign (Dnastar) and another Perl script was used
25 to create a minority consensus sequence representing each serotype. Finally, consensus

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3 1 sequences for all serotypes were aligned in MegAlign for visual identification of conserved
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6 2 sites for primer design.

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8 3 The anchored Pan Dengue RT-PCR included the 3' genome end cDNA synthesis/reverse
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10 4 transcription (3'RT), the second strand synthesis (SS), and the tag primed PCR amplification
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12 5 (TP-PCR). Primers for the 3'RT and SS steps were designed from the created dengue
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14 6 alignment. These included 3'RT primers: 3'RT-p1, 3'RT-p2A, 3'RT-p2B, 3'RT-p3A, 3'RT-
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16 7 p3B, 3'RT-p3C (Table 1), and SS primers: SS-p1A, SS-p1B, SS-p1C, SS-p2A, SS-p2B, and
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18 8 SS-p2C (Table 1). A mutual artificial tag primer sequence was included on the 5' end of both
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20 9 the 3'RT and SS primers for the TP-PCR amplification. The TAG primer (Table 1) was used
21
22 10 as previously described (11).
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27 11 The primers for specific SYBR Green real-time PCR quantitation of the cultured dengue
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29 12 virus strains were designed with Primer Express 2.0 (Applied Biosystems, Foster, City, CA,
30
31 13 USA) near the 3' genome end (QSP-assays: QSP-D1U (forward) and QSP-D1L (reverse),
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33 14 QSP-D2U and QSP-D2L, QSP-D3U and QSP-D3L, QSP-D4U and QSP-D4L, in Table 1).
34
35 15 Real-time PCR primers for broad dengue virus RNA quantitation in clinical samples (QST-
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37 16 assays: QST-D1U (forward) and QST-D1L (reverse), QST-D2AU and QST-D2AL, QST-
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39 17 D2BU and QST-D2BL, QST-D3U and QST-D3L, QST-D4U and QST-D4L, in Table 1), were
40
41 18 designed from the serotype consensus sequences using Oligo (Molecular Biology Insights,
42
43 19 Cascade, CO, USA). These were primarily designed for detection of all known dengue virus
44
45 20 strains but were not completely serotype specific. For quantification of 18S ribosomal RNA
46
47 21 (NR_003286) in serum, primers HUM18SU (forward) and HUM18SL (reverse) were
48
49 22 designed, and for 28S ribosomal RNA (NR_003287) primers HUM28SU (forward) and
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51 23 HUM28SL (reverse) were designed. All primers, including random hexamers, were ordered
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53 24 from Eurofins MWG Operon (Ebersberg, Germany).
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1 RNA extraction and preparation of sample material

2 For testing of the assay using spiked serum, both serum RNA and viral RNA were extracted
3 by adding Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA) to 125 μ l sample following
4 the manufacturer's instructions. After phase separation by centrifugation the aqueous phase
5 was transferred to the QIAamp MinElute Viral Spin Kit (Qiagen, Hilden, Germany). It was
6 mixed with binding buffer, applied on one MinElute spin column, and eluted to 30 μ l. To
7 increase the level of human background nucleic acid, by 2-fold and 10-fold, respectively,
8 larger volumes of the aqueous phase from serum were extracted on one spin column by
9 applying the maximum volume repeatedly before elution to 30 μ l. Typically, 1 μ l extracted
10 serum RNA and 1 μ l viral RNA of known concentration were mixed for 3'RT cDNA
11 synthesis or one-step anchored Pan Dengue RT-PCR. The 2-fold concentration contained
12 approximately 1.7×10^7 copies/ μ l of 18S rRNA (2.0×10^6 copies/ μ l serum) and
13 6.8×10^6 copies/ μ l of 28S rRNA (8.2×10^5 copies/ μ l serum).

14 For evaluation of possible dual infections of different dengue serotypes, 0.5 μ l of diluted
15 viral RNA for each serotype was added together with 1 μ l serum RNA.

16 Clinical samples from patients with dengue infections were extracted by MagAttract Viral
17 RNA M48 Kit (Qiagen) in a Genovision GenoM48/BioRobot M48 (Qiagen).

19 3' genome end cDNA synthesis

20 For the 3' genome end targeted cDNA synthesis (3'RT) the Superscript III reverse
21 transcriptase kit (Invitrogen) was used. In general, the manufacturer's instructions were
22 followed, but after optimization 500 nM of 3'RT primer, alternatively 3'RT1-p1, 3'RT-p2 mix
23 (50% each of 3'RT-p2A and 3'RT-p2B) or 3'RT-p3 mix (33% each of 3'RT-p3A, 3'RT-p3B
24 and 3'RT-p3C), was used in a 10 μ l reaction volume with 2 μ l extracted RNA. After adding
25 the enzyme mixture, the reaction was incubated at 57°C for 1 hour in a 2700 PCR system

1 (Applied Biosystems). For the comparison of cDNA synthesis efficiency, the specific reverse
2 primer (QSP-D2L, 100 nM) and random hexamer primers were used following the
3 manufacturer's instructions.

4 5 **Second strand synthesis and tag primed PCR**

6 Using the 3'RT cDNA as template, the second strand synthesis (SS) and tag primed PCR (TP-
7 PCR) were performed on a Tgradient thermocycler (Whatman Biometra, Goettingen,
8 Germany), in a 25 µl reaction volume including 1.25 units Platinum Taq (Invitrogen), 2.5 µl
9 10×buffer, 3 mM MgCl₂, 400 µM dNTP (Invitrogen), 2 µM tag primer (TAG), 0.2 µM SS
10 primers and 2,5 µl cDNA. SS primers were the SS-p1 mix (33% each of SS-p1A, SS-p1B and
11 SS-p1C) or the SS-p2 mix (33% each of SS-p2A, SS-p2B, and SS-p2C). Following 2 minutes
12 at 95°C, 5 cycles of touchdown PCR were performed for second strand synthesis. These
13 included 1 minute annealing, starting at 65°C and decreased by 1 degree per cycle, 45
14 seconds at 72°C and 30 seconds at 95°C. Fifty cycles of TP-PCR amplification followed,
15 with 95°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute. The program was
16 completed with 72°C for 10 minutes.

17 18 **One-Step anchored Pan Dengue RT-PCR**

19 The SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase Kit
20 (Invitrogen) was used for combination of the 3'RT, SS and TP-PCR in one reaction. 1 µl of
21 RT/Platinum Taq Mix, 12.5 µl of 2×reaction mix, 2.5 units Platinum Taq (Invitrogen), 1.4
22 mM MgSO₄, 200 µM dNTP, 2 µM tag primer (TAG), 0.2 µM 3'RT primer (RT-p1), 0.2 µM
23 SS primers (SS-p1 mix), and 2 µl RNA was added to a 25 µl reaction. A program was run on
24 a Tgradient thermocycler starting with 65°C for 1 minute and 57°C for 30 minutes followed
25 by the same program as for the SS and TP-PCR.

1 SYBR Green real-time PCR

2 SYBR Green real-time PCR was run on the ABI 7900HT Sequence Detection System
3 (Applied Biosystems) in a Veriti 96-well Fast plate (Applied Biosystems) to quantify both
4 template and amplicons during development of the anchored Pan Dengue RT-PCR (QSP-
5 assays) as well as for detection and approximate quantitation of viral RNA in serum samples
6 (QST-assays). A standard program including dissociation stage was used. The amplification
7 efficiencies (E) of the real-time PCR assays (QSP-assays) for the four cultured dengue virus
8 strains were 91.0% (DENV-1, QSP-D1U and QSP-D1L), 96.2% (DENV-2, QSP-D2U and
9 QSP-D2L), 87.6% (DENV-3, QSP-D2U and QSP-D2L) and 89.3% (DENV-4, QSP-D4U and
10 QSP-D4L), respectively. This was established on random hexamer cDNA from extracted
11 virus RNA and in addition the DENV-2 assay was tested on a plasmid containing a fragment
12 of DENV-2 New Guinea C covering position 5371 to 3' genome end. The corresponding
13 amount of RNA in a SS-TP-PCR reaction was calculated using the formula of $(1+E)^{(40-C_t)}$
14 $^{C_t}/(\text{volume of cDNA in real-time PCR reaction}) \times (\text{volume of cDNA added})$. The QSP-assays
15 were used in the development to quantify template in form of dengue 3' genome end cDNA
16 and the generated anchored Pan Dengue RT-PCR products that were not purified but diluted
17 10^6 times. Both the QSP-assays and the QST-assays were tested for background amplification
18 on random hexamer cDNA from negative serum RNA. Standard protocols were also used for
19 the QST-assays and the specific real-time PCR assays for quantitation of human 18S and 28S
20 ribosomal RNA in serum. For detection and approximate quantitation of random hexamer as
21 well as 3' genome end cDNA from the clinical samples, both the QST-assays and the QSP-
22 assays were run in parallel and the results were compared.

24 Fast Sanger sequencing

25 Before Fast Sanger sequencing, the products generated by the anchored Pan Dengue RT-

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3 1 PCRs were purified with the MinElute PCR Purification Kit (Qiagen), or initially with the
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5 2 MinElute Gel Extraction Kit (Qiagen) from a 2% agarose gel. Then, 2 pmol 3' RT-p1 primer,
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7 3 0.5 μ l BigDye Terminator v1.1 Cycle Sequencing mix (Applied Biosystems), 1.5 μ l
8
9 4 5 \times sequencing buffer and 2 μ l purified PCR product diluted 10 times were added to a 10 μ l
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11 5 sequencing PCR reaction. The reactions were run in a Veriti 96-well Fast plate on a PCR
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13 6 9800 system (Applied Biosystems) with a program in FAST ramping mode, starting with
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15 7 96°C for 1 minute followed by 25 cycles of 96°C for zero seconds, 50°C for zero seconds and
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17 8 60°C for 30 seconds. The resulting products were purified using the BigDye XTerminator
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19 9 Purification Kit (Applied Biosystems) according to the manual, except for vortexing 5
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21 10 minutes at 2200 rpm on a Eppendorf Mixmate (Eppendorf AG, Hamburg, Germany), and run
22
23 11 on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) with the UltraSeq36 module.
24
25 12 The generated raw data were analyzed with Sequence Scanner v1.0 software, and the
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27 13 resulting sequences were Blast-searched (NCBI-GenBank) and aligned against the minority
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29 14 consensus sequences of the four dengue serotypes with MegAlign.
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1 **Results**

2 **Development of the anchored Pan Dengue RT-PCR**

3 The objective of this study was to develop an assay combining a RT-PCR that targets all four
4 dengue serotypes (Pan Dengue) in one reaction, with a rapid Sanger sequencing protocol. The
5 developed anchored Pan Dengue RT-PCR included three steps: a highly specific cDNA
6 synthesis (3'RT), a less stringent second strand synthesis (SS), and amplification of the
7 generated dsDNA complexes by a tag primed PCR (TP-PCR). First a two-step RT-PCR
8 protocol was developed, with a separate reaction for cDNA synthesis followed by SS and TP-
9 PCR in a second reaction (SS-TP-PCR). Secondly, the separate reactions were also combined
10 in a one-step RT-PCR protocol to save time and work. The cDNA synthesis was optimized
11 starting from the manufacturer's instructions and the second reaction was developed from a
12 previously used protocol (11). The complex setup was thoroughly evaluated by varying
13 different parameters and quantifying the amounts of 3'RT cDNA and SS-TP-PCR amplicons
14 produced using virus strain specific real-time PCR. Selected amplification products were
15 sequenced to verify the specificity. The DENV-2 New Guinea C strain was diluted to
16 plausible levels for clinical samples, i.e. 10^3 - 10^4 copies/ μ l, and mixed with 2-fold
17 concentrated human serum RNA for the majority of the development work but the protocols
18 were also evaluated on cultured strains representing the other serotypes.

19 Based on the published dengue nucleic acid sequences, the outermost 3' genome ends
20 seemed highly conserved and possible to target for cDNA synthesis (Table 1, Fig. 2). The
21 identified site was used to design several different primers. The sequence variation increased
22 further from the dengue genome 3' end, and due to the importance of avoiding mismatches at
23 the first five positions at the 3' end of the primer, a short as possible oligonucleotide was
24 preferred. The shorter primer, 3'RT-p1, was degenerated at the sixth and seventh positions
25 from the 3' primer end to accommodate differences in the DENV-4 sequences (Fig. 2). As an

1 alternative, two separate primers were also designed for the same position (3'RT-p2 mix).
2 Additional primers were designed by further extension from the 3' genome end (3'RT-p3
3 mix).
4 The primers/primer mixes were compared for cDNA synthesis using the protocol for specific
5 primers in the Superscript III manual, with the exception of half the reaction volume, to
6 increase the concentration of cDNA added to the subsequent SS-TP-PCR reaction. This
7 included a 55°C annealing/synthesis temperature for 1 hour with 100 nM primer
8 concentration, after denaturation and addition of the enzyme mix. The preferred 3'RT-p1
9 primer produced as much or more cDNA as the two primer mixes and was selected for further
10 optimization for the highest possible stringency, in order to minimize possible background
11 amplification, while still preserving an acceptable cDNA production. Taking the 2.5 minute
12 half-life at 60°C of Superscript III into account, different temperatures between 55 to 60°C
13 were tested, both in a separate 30 seconds annealing step inserted before a 1 hour synthesis
14 step at 55°C, as well as in a single annealing/synthesis step. It was found, that a single
15 annealing/synthesis step at 57°C for 1 hour gave the optimal result in terms of both
16 specificity and cDNA production when performing repeated testing on the spiked serum RNA.
17 In parallel, different primer concentrations were compared and an increase from 100 to 500
18 nM was ultimately selected. For reference, a protocol used widely for random hexamer
19 cDNA synthesis produced up to 8 times more cDNA than the developed 3'RT protocol. The
20 higher amount of cDNA produced for the random hexamer protocol was probably due to both
21 cDNA synthesis from viral RNA lacking the outermost part of the 3' genome end, which was
22 not targeted by the 3'RT-p1 primer, as well as a higher stringency for the 3'RT protocol.
23 With the main objectives of high amplification, short assay time, and low cost, additional
24 parameters were evaluated one at-a-time for the subsequent SS-TP-PCR reaction. Six
25 degenerated primers were designed for the second strand synthesis, from sites conserved

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3 1 between the four serotypes at approximately 130 (SS-p1 mix) and 500 nucleotides (SS-p2
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5 2 mix) from the 3' end, respectively (Table 1). Comparison of the two mixes of primers, as well
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8 3 as individual primers, demonstrated equal amplification, but the sequences were longer and
9
10 4 with better quality for the SS-p1 mix. A 0.2 μM concentration of the SS-primer mix was
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12 5 sufficiently high to compensate for the relatively high degeneracy of the primers. Both
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14 6 different annealing temperatures as well as different numbers of touchdown cycles were then
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16 7 tested for the SS step. It was found that five cycles starting from 65°C annealing gave the
17
18 8 best result in terms of specificity, amplification products, and sequence quality. For the TP-
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20 9 PCR step the artificial tag primer sequence (TAG) had been used previously (11).
21
22 10 Nevertheless, the optimal concentration of the TAG primer, 2 μM , and annealing temperature,
23
24 11 58°C, were determined to give the best result in terms of both amplification and specificity.
25
26 12 Comparison showed that 70 cycles gave two times more amplification than 50 cycles, but
27
28 13 longer and better Sanger sequences were obtained for 50 cycles in a considerably shorter time.
29
30 14 After testing different concentrations, it was found that 1.25 units of Platinum Taq enzyme
31
32 15 and 3 mM Mg^{2+} produced the best result for the combined SS-TP-PCR reaction.
33
34 16 The 3'RT and SS-TP-PCR reactions were transformed with minor modifications to a one-
35
36 17 step RT-PCR format including more or less the same reagents in one single reaction. Despite
37
38 18 including the Superscript III enzyme from the beginning, it still proved possible to include an
39
40 19 initial incubation at 65°C for one minute to resolve potential secondary structures and
41
42 20 increase the stringency. Upon this followed the synthesis step at 57°C for which 30 minutes
43
44 21 incubation gave the best result. An addition of 2.5 units Platinum Taq to the reaction, together
45
46 22 with the kit enzyme mix, increased the amplification approximately 10 times.
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59 24 **Optimization of the whole procedure for the assay**
60 25 The whole procedure for the assay was optimized for completion in as short a time as

1 possible, while preserving an acceptable performance and work effort. The initial sample
2 extraction on spin column was compared to robotic extraction, which displayed a similar
3 result but with larger elution volumes and lower flexibility. The anchored Pan Dengue RT-
4 PCR products were purified on spin columns, prior to the rapid sequencing PCR, which was
5 found to be better without purification or gel extraction. For the subsequent rapid sequencing
6 PCR it was found that 0.5 μ l BigDye Terminator mix was sufficient. The resulting procedure
7 from sample extraction to sequence analysis could be completed within five hours, including
8 six or seven working steps if a one-step or two-step RT-PCR format was used. The whole
9 Fast Sanger sequencing step with 16 samples only took about 1.5 hours starting from the
10 rapid sequencing PCR until completion of the sequence analysis.

12 **Evaluation of the detection limit for the anchored Pan Dengue RT-PCR protocols**

13 To analyze the limit of detection, both the one-step and two-step RT-PCR based protocols
14 were tested on dilutions of viral RNA spiked in serum RNA (Table II, Fig. 2). Initially,
15 DENV-2 was tested, and the detection level was established after repeated measurements.
16 Based on this result the other serotypes were evaluated. Table II displays real-time PCR
17 quantification of 3'RT-p1 cDNA and of amplicons generated by both the one-step and two-
18 step anchored Pan Dengue RT-PCRs. For the two-step format the same 3'RT-p1 cDNA was
19 used as template for the SS-TP-PCR as well as for the real-time PCR quantitation. For
20 evaluation of the one-step format 3'RT-p1 cDNA synthesis was carried out using the same
21 tube of RNA.

22 The detection limit for the two-step RT-PCR format testing the different serotypes was
23 between 11 and 31 copies of 3'RT-p1 cDNA added to the SS-TP-PCR reaction or 44-124
24 copies of viral RNA added to the 3'RT-p1 cDNA reaction. This corresponds to between 5-15
25 copies of viral RNA/ μ l serum for the sample extraction used. The one-step RT-PCR format

1 displayed a less consistent result contributing to a range of 100-500 copies extracted viral
2 RNA added to the reaction or 12-60 copies/ μ l serum. Both formats of the assay performed
3 best on the DENV-1 strain and less well on the DENV-4 strain. The two-step RT-PCR format
4 displayed a better detection limit, but for the positive results both the one-step and the two-
5 step RT-PCR protocols amplified the RNA/cDNA more than 1 million times.

6 Looking at produced sequences, they started 20-40 basepairs off the sequencing primer, and
7 had an acceptable quality, typically ranging from 440 to 520 basepairs, which covers most of
8 the expected amplicons (Fig. 2). Blast match identity versus the published sequences of these
9 strains was over 90% which gave clear distinction between different serotypes.

10 The capacity of this assay to resolve dual infections was evaluated on serum RNA spiked
11 with viral RNA from two different serotypes at different amounts and run with the two-step
12 RT-PCR protocol. The intention was to look for dual peaks in informative positions, but in
13 most cases only one strain could be identified, even when using equal amounts of RNA.

15 **Evaluation on confirmed clinical samples**

16 Serum samples from ten patients were tested by both the one-step and two-step RT-PCR
17 based formats of the anchored Pan Dengue RT-PCR assay (Table III, Fig. 2). RNA from
18 cultured strains and serum was used as positive and negative controls, respectively. The
19 samples had been tested with multiplex endpoint RT-PCR or serotype specific real-time RT-
20 PCR previously, confirming the existence of viral RNA. To obtain a more recent reference
21 due to RNA degradation and for comparison of detection limits, the samples were run again
22 by real-time RT-PCR, in parallel with the anchored Pan Dengue RT-PCR assay. For each
23 sample, SYBR Green real-time PCR was run on both the 3' RT cDNA as well as on random
24 hexamer cDNA, to provide a universal comparison with real-time RT-PCR based methods
25 and to assess the proportion of viral RNA missing the 3' genome end. Both the strain specific

1 (QSP-assays) and the serotype specific assays (QST-assays) were run, to ensure a positive
2 result. The lowest Ct values produced for the different assays were selected and displayed in
3 Table III. With most samples this proved to be the strain specific assay even if the differences
4 were small.

5 The two-step RT-PCR format of the anchored Pan Dengue RT-PCR gave positive results for
6 all ten samples, while the one-step RT-PCR format only produced positive results for three
7 samples (D1_CS1, D2_CS1, and D2_CS3). However, a comparison of these results to the
8 real-time PCR Ct values obtained for the 3'RT cDNA, showed a relatively good agreement
9 with the established detection limits. The three samples positive by the one-step RT-PCR
10 format had considerably lower Ct values for both the 3'RT cDNA as well as the random
11 hexamer cDNA, indicating Dengue RNA levels at $>10^6$ copies/ml serum. For the other
12 samples the Ct values indicated levels of viral RNA around the lower detection limit for the
13 two-step RT-PCR format of the anchored Pan Dengue RT-PCR, and clearly below the one-
14 step RT-PCR format. Two samples representing different serotypes and consequently
15 measured by different real-time PCR assays, D1_CS2 and D2_CS2, even had Ct values
16 similar to negative serum RNA for the 3'RT cDNA (Ct 39 for DENV-1, Ct 37 for DENV-2,
17 and Ct 40 for DENV-3), and could therefore not be considered as detectable by the SYBR
18 Green real-time PCR. It indicated that the two-step RT-PCR format of the anchored Pan
19 Dengue RT-PCR has a detection limit that at least is equal to a real-time RT-PCR when using
20 the same cDNA synthesis protocol.

21 In comparison to the protocol for random hexamer cDNA synthesis, the 3'RT cDNA
22 produced between 1 and 8.4 units higher Ct value for the ten samples. A large difference
23 indicates a high proportion of viral RNA missing the 3' genome end. It could, in most cases,
24 be attributed to repeated freeze-thawing of the samples which could degrade the 3' genome
25 end of the viral RNA.

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3 1 For the DENV-2 samples (D2_CS1, D2_CS2, D2_CS3) the 3'RT cDNA was tested with the
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5 2 QSP-assay (QSP-D2U and QSP-D2L) and with the QST-assay (QST-D2BU and QST-D2BL).
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7 3 But for the random hexamer cDNA there was an additional QST-assay used (QST-D2AU and
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9 4 QST-D2AL), which targeted positions further into the genome and provided an extra
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11 5 reference, in case of considerable degradation of the viral RNA near the 3' genome end. The
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13 6 different DENV-2 real-time PCR assays were compared on a cloned fragment covering the
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15 7 sites for all three assays, which showed that the QSP-assay produced between 2 and 3 units
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17 8 lower Ct values than both the other two assays. Taking this in consideration, the equal or
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19 9 slightly lower Ct values obtained for the assay targeting positions located further into the
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21 10 genome indicates the presence of approximately 2-8 times more cDNA from that part of the
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23 11 genome.
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25 12 The length of the produced sequences varied between 130-450 bp, which was shorter than
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27 13 for the cultured strains, but still sufficient for identification of the serotype. In general, the
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29 14 samples with higher amounts of viral RNA gave longer sequences.
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1 Discussion

2 From a broader perspective, the strategy for the anchored Pan Dengue RT-PCR should also
3 be applicable for other RNA viruses. The assay not only incorporates a new faster Sanger
4 sequencing protocol for dengue virus RNA detection and identification, but is designed to
5 have an ability capacity to detect new strains of dengue viruses. Its ability to screen all four
6 dengue serotypes in one reaction saves time and work which is particularly important in cases
7 of severe disease when differential diagnosis and distinct sequence verification is warranted.
8 In these cases it might not be sufficient to run standard end-point RT-PCR or real-time RT-
9 PCR only. Instead further verification of RT-PCR products by Sanger sequencing is needed.
10 The integrated procedure of this assay is then comparable in cost and easier to perform.
11 The design of the anchored Pan Dengue RT-PCR should provide automatically a better
12 detection capacity of new strains compared to the standard RT-PCR protocols, but the
13 complexity of the design makes it important to evaluate the detection limit of the assay
14 carefully. It was evaluated primarily using viral RNA from cultured Dengue strains spiked in
15 RNA extracted from serum to obtain a valid background. This demonstrated a higher
16 detection limit for the two-step format of the anchored Pan Dengue RT-PCR than expected
17 for RT-PCR methods used routinely. However, this is not unexpected considering the
18 requirement of viral RNA with intact 3' genome ends for the anchored Pan Dengue RT-PCR.
19 The verification on the set of clinical samples on the other hand actually indicated a relatively
20 good performance by this assay. If the same protocol for cDNA synthesis, targeting the 3'
21 genome end, is used in combination with real-time PCR detection, the result is about the
22 same as for the two-step based format of the assay. In samples with very extensive
23 degradation of the viral RNA there might still be a problem to get a positive result. In the set
24 of samples investigated, the differences in Ct values of the 3'RT cDNA compared to the
25 standard random hexamer cDNA ranged from 1 to over 8 units, but the larger differences

1 were found for samples which had been freeze-thawed repeatedly. In a completely fresh
2 carefully handled sample, more than a difference of 3 Ct values would not be expected. In
3 terms of real-time PCR quantified random hexamer cDNA, it would correspond to 992 copies
4 (124 copies \times 2³) of DENV-4 RNA added to the 3'RT cDNA reaction or 113 copies of virus
5 RNA per μ l serum. Considering the high amounts of virus RNA associated with acute phase
6 samples [Lindgren et al., 2005], even the one-step RT-PCR format of the assay should be
7 sufficient in many instances, while mutations inhibiting primer binding might increase the
8 detection limit of a conventional RT-PCR involving two specific primers considerably. The
9 one-step RT-PCR format of the assay was found to amplify the virus cDNA to the same
10 extent as the two-step format, but more RNA was needed to start the amplification, which
11 indicates that the cDNA synthesis was less efficient. However, this is to be expected for such
12 a complex reaction and it might still be advantageous to use this format in cases of patients
13 with vague symptoms and a need to screen for several different pathogens by different assays.
14 The capacity for improved detection of viral RNA over the RT-PCR protocols used routinely
15 could obviously not be tested on these already confirmed samples. We have only encountered
16 a few cases of acute-phase samples that have been negative initially but later were confirmed
17 as positive for Dengue viral RNA using other RT-PCR protocols or microarray assays
18 [Nordström et al., 2005]. However, if possible, the method described above will be compared
19 with the different assays used routinely, including real-time RT-PCR and NS1 antigen tests,
20 on a larger set of samples for comparison of the detection capacity.
21 For other types of samples than human serum, or for development of completely new assays,
22 a more extensive evaluation must be conducted since the specificity of this assay is dependent
23 primarily on correct binding of the primer for cDNA synthesis. Non-specific binding of the
24 other primers could inhibit the amplification but not the sequencing result. The serum used in
25 this study to create spiked samples for extensive evaluation of the assay was a mixture from

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3 1 several individuals which should create a broad human nucleic acid background. An attempt
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5 2 was made to increase the amounts of serum RNA to twice that normally expected for the
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7 3 extraction procedure used, to enhance further possible background influence during the
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9 4 development work. When the amount of serum RNA was increased to about ten times the
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11 5 expected concentration, it was observed that human sequences were produced when 10^3
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13 6 copies of viral RNA were added to the 3'RT cDNA reaction. Therefore, in sample types with
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15 7 large amounts of human nucleic acid the amount of viral RNA must be very high to compete
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17 8 with the background.
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22 9 The capacity for resolution of dual infections was tested briefly with negative results.
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25 10 Usually, however, at least one strain in the sample can be identified, and outside the endemic
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27 11 regions dual infections are unlikely. Instead, the ability to detect new dengue strains is more
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29 12 valuable. In rare cases of dual infections, hybridization based assays, such as the Flavivirus
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31 13 microarray developed previously [Nordström et al., 2005], perform better.
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3 **1 Acknowledgements**
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14 6 linguistic advice.
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For Peer Review

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30 prospective clinical study on the use of reverse transcription-polymerase chain
31 reaction for the early diagnosis of Dengue fever. *J Mol Diagn* 8(5):613-616; quiz 617-
32 620.

1 TABLE I. Primer sequences with reference position in the cultured strains.

Name	Primer sequences ^a	Position (Accession No.)
TAG	<u>GTTTCCCAGTCACGATC</u>	
3'RT-p1	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGRWTC AAC	10717-10734 (EU848545)
3'RT-p2A	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGGATCAAC	10717-10734 (EU848545)
3'RT-p2B	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGATTCAAC	10717-10734 (EU848545)
3'RT-p3A	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGATTCAACVGCACCA	10710-10734 (EU848545)
3'RT-p3B	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGATTCAACAGSACCA	10710-10734 (EU848545)
3'RT-p3C	<u>GTTTCCCAGTCACGATC</u> AGAACCTGTTGGATCAACAACACCA	10710-10734 (EU848545)
SS-p1A	<u>GTTTCCCAGTCACGATC</u> AYTACATGMCWKYVATGAARAGAT	10213-10236 (EU848545)
SS-p1B	<u>GTTTCCCAGTCACGATC</u> GSAAWGARRADTWYVYRGAYTACATG	10195-10220 (EU848545)
SS-p1C	<u>GTTTCCCAGTCACGATC</u> HCARGTBAGR WVSVCTBATHGGSAA	10175-10198 (EU848545)
SS-p2A	<u>GTTTCCCAGTCACGATC</u> AGMKGTTAKWGGARACCCC	10595-10614 (EU848545)
SS-p2B	<u>GTTTCCCAGTCACGATC</u> NNNNNNGACYAGMKGTTAKWGGA	10591-10607 (EU848545)
SS-p2C	<u>GTTTCCCAGTCACGATC</u> NNNNNNNNNNGACYAGMKGTTA	10591-10602 (EU848545)
QSP-D1U	ACAACGCAGCAGCGGG	10539-10554 (EU848545)
QSP-D1L	TCTAGTCCTTACCACCAGGGTACAG	10574-10597 (EU848545)
QSP-D2U	AGACCCCTCCCTTACAAATCG	10511-10531 (AF038403)
QSP-D2L	TGGTCTTTCCCAGCGTCAAT	10625-10644 (AF038403)
QSP-D3U	CCTTGCAAAGGACTAGAGGTTATAGG	10545-10570 (M93130)
QSP-D3L	TGCTGTTTTTGTGTTGCGGG	10577-10595 (M93130)
QSP-D4U	GGTGGAAGGACTAGAGGTTAGAGGAG	10507-10532 (AY947539)
QSP-D4L	CAATATGCTGTTTTGTGTTGGG	10538-10560 (AY947539)
QST-D1U	GAGACCCCSGCRYA	10606-10620 (EU848545)
QST-D1L	CTKTGCCTGGAWTGATGCTG	10673-10692 (EU848545)
QST-D2AU	TCAAAGACCAACMCCAAGAGG	9309-9329 (AF038403)
QST-D2AL	CATCTCCACTGATDCCATTCTTG	9536-9560 (AF038403)
QST-D2BU	CGCRTGGYGTAGTGGACT	10482-10499 (AF038403)
QST-D2BL	TGGTCTTTCCCAGCGTCAAT	10625-10644 (AF038403)
QST-D3U	GAGACCCCSGCRYA	10570-10584 (M93130)
QST-D3L	GGTCTCTCCCAGSGTCAATA	10596-10615 (M93130)
QST-D4U	GAWGYCAGGAKGAAGCTGTACT	10482-10503 (AY947539)
QST-D4L	GCGCTCTGTGCCTGGATTGATG	10599-10691 (AY947539)
HUM18SU	CCTGCGGCTTAATTTGACTC	1230-1249 (NR003286)
HUM18SL	GCTCCACCAACTAAGAACGG	1336-1355 (NR003286)
HUM28SU	CGATGTCGGCTCTTCTATC	4417-4436 (NR003287)
HUM28SL	ACCATGGCAACAACACATCA	4539-4558 (NR003287)

2 ^a The artificial 5' tag primer sequence is underlined. Primers QST-D1U and QST-D3U have
3 identical sequence, as well as primers QSP-D2L and QST-D2BL.

1 TABLE II. The limit of detection for the anchored Pan Dengue RT-PCR protocols tested on
2 cultured strains.

Serotype	Two-step RT-PCR				One-step RT-PCR		
	Copies of vRNA ^a	Copies of cDNA ^b	Match position (Accession No.) ^d	ID (%) ^e	Copies of vRNA ^c	Match Position (Accession No.) ^d	ID (%) ^e
DENV-1	44	11	10176-10697 (EU848545)	94	100	10558-10697 (EU848545)	99
DENV-2	100	25	10338-10653 (AF038403)	88	351	10456-10687 (AF038403)	94
DENV-3	56	14	10219-10662 (M93130)	100	100	10208-10641 (M93130)	98
DENV-4	124	31	10205-10662 (AY9447539)	95	500	10268-10617 (AY947539)	99

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4 ^a Number of copies of vRNA added to the 3'RT reaction for the two-step anchored Pan
5 Dengue RT-PCR; ^b Number of copies of cDNA added to the reaction including the SS and
6 TP-PCR steps; ^c number of copies of vRNA added to the one-step anchored Pan Dengue RT-
7 PCR reaction; ^d Blast result for generated sequence; ^e Blast match identity. Adding 2 μ l
8 sample containing 100 copies of viral RNA to the 3'RT reaction corresponds to 12 copies of
9 viral RNA/ μ l serum with the current extraction protocol.

1 TABLE III. Results for testing of confirmed clinical samples.

Tested sample	Serotype	Two-step RT-PCR Blast result ^{a, b}		One-step RT-PCR Blast result ^{a, b, c}		Real-Time PCR (Ct) ^d	
		Accession No. (Match position)	ID (%)	Accession No. (Match position)	ID (%)	Rand6 cDNA ^{e, f, g}	3' RT cDNA ^c
D1_CS1	DENV-1	10178-10659 (FJ850068)	99	10498-10663 (FJ906728)	100	21,3	27,3
D1_CS2	DENV-1	10152-10679 (FJ850087)	99	nd		37,8	nd
D1_CS3	DENV-1	10608-10696 (EU848545)	97	nd		26,7	35,1
D1_CS4	DENV-1	10330-10607 (GQ868569)	98	nd		31	36,4
D2_CS1	DENV-2	10208-10606 (FJ432724)	98	10217-10666 (FJ639705)	98	25,3* / 24,0**	27,7
D2_CS2	DENV-2	10277-10687 (FN429894)	99	nd		30,2* / 29,3**	37,2
D2_CS3	DENV-2	10415-10652 (FJ432724)	98	10312-10648 (FJ432724)	100	27,3* / 27,4**	28,3
D3_CS1	DENV-3	10491-10656 (GQ868576)	99	nd		30,2	34,6
D3_CS2	DENV-3	10354-10632 (GU131952)	98	nd		31,8	36,4
D3_CS3	DENV-3	10242-10654 (FJ882575)	100	nd		32,3	36,2

^a Accession No., GenBank nucleotide sequence accession number; ^b ID, Blast match identity;

^c *nd*, not detected; ^d Lowest Ct values obtained for the dengue Real-Time PCR assays; ^e

Rand6, random hexamer primed cDNA; ^f *, Ct value for primers located near the 3' genome

end: QST-D2BU and QST-D2BL or QSP-D2U and QSP-D2L; ^g **, Ct value for primers

located at position 9309-9560: QST-D2AU and QST-D2AL.

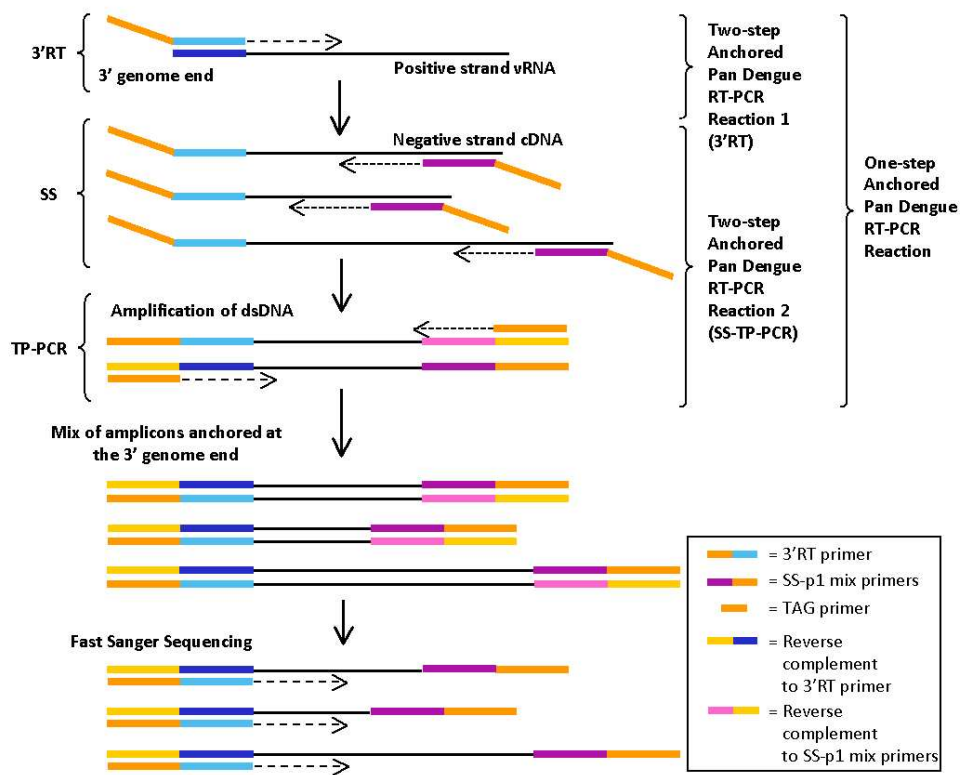
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3 **1 Legends**
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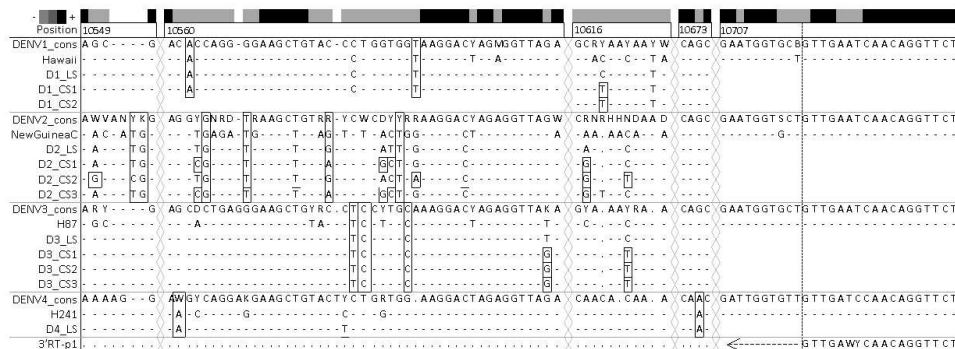
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17 6 Fig. 2. Sequence alignment.
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19 7 Reference positions in EU848545; boxes indicate informative sequence features; '.', gap; '-',
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21 8 equal to serotype consensus; DV1_cons, DENV-1 consensus; Hawaii, the published dengue 1
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23 9 Hawaii sequence (EU848545); D1_LS, sequencing result for the Hawaii strain; D1_CS,
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26 10 clinical sample; the same manner of designation is then used for all serotypes including the
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28 11 cultured strains; 3'RT-p1, the selected 3' genome end cDNA primer.
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Schematic outline of the assay.



Sequence alignment.

Reference positions in EU848545; boxes indicate informative sequence features; '.', gap; '-', equal to serotype consensus; DV1_cons, DENV-1 consensus; Hawaii, the published Dengue 1 Hawaii sequence (EU848545); D1_LS, sequencing result for the Hawaii strain; D1_CS, clinical sample; the same manner of designation is then used for all serotypes including the cultured strains; 3'RT-p1, 3' genome end cDNA primer.