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Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma

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Abstract

Dengue is mosquito-borne virus infection that annually causes ~50 million clinically apparent cases worldwide. An internally controlled one-step real-time multiplex RT-PCR assay was developed for detection and quantitation of DENV RNA in plasma sample by using specific primers and fluorogenic TaqMan probes. All primers and probes targeted sequences near the 3′ end of the NS5 gene. The method comprised two multiplex assays and was validated for sensitivity, specificity, linearity, reproducibility and precision. An internal control template was spiked into each clinical specimen to provide quality assurance for each experimental step. The assay allowed for detection of between 0.5 and 3 infectious particles per mL, is rapid and has been operationally characterized in 287 Vietnamese dengue patients from two therapeutic intervention trials at the Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam.

Keywords

Dengue virus; One step real time multiplex RT-PCR; Validation

1. Introduction

Dengue is a mosquito-born virus disease that is caused by four phylogenetically and antigenically distinct types of dengue virus. There are an estimated 50 million dengue cases per annum with tropical and sub-tropical countries particularly affected (WHO, 2009). The four dengue serotypes (DENV-1–4) produce symptoms that range from mild fever to the

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2011.08.002.

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more severe syndromes involving systemic vascular leakage, haemorrhage and/or organ dysfunction.

In most endemic settings, the diagnosis of dengue is made on clinical grounds alone. In some settings, this is supported with serology to detect DENV reactive IgM or with antigen detection of the virus-expressed NS1 protein that circulates in blood during the acute phase. Both IgM and NS1 detections are robust diagnostic methods that nonetheless have well-described limitations. For example, IgM is often not detectable until the 5th day of illness, and therefore is often not useful for early patient triage (Hu et al., 2011; Shu and Huang, 2004). NS1 can be detected in the first few days of infection, but is not detectable in all patients in part because of differences in sensitivity between commercial assays (Dussart et al., 2008; Hang et al., 2009) but also because of serotype-dependent differences in NS1 antigenemia (Duyen et al., 2011; Simmons et al., 2007).

RT-PCR based detection of DENV RNA in plasma or serum allows for early diagnosis of dengue and quantitation of virus RNA concentrations. Relatively high DENV RNA levels in the first 2–3 days of illness have been repeatedly associated with more severe clinical syndromes, suggesting that quantification of early dengue viremia levels has prognostic value (Hoang et al., 2010; Vaughn et al., 2000, 1997). The disadvantages of RT-PCR for diagnosis in endemic settings are the requirement for relatively expensive reagents, equipment and specialized training of laboratory staff. However, the absence of specific interventions for the treatment of dengue (e.g. an anti-viral drug or immunomodulatory therapy) undermines the demand in resource limited settings for relatively expensive diagnostics such as RT-PCR; in these settings a clinical diagnosis coupled with routine laboratory findings is often the basis for patient management. Nonetheless there are compelling reasons to develop and validate sensitive and specific RT-PCR assays for the diagnosis of dengue. First, such assays are likely to continue to feature in studies of pathogenesis (Rico-Hesse et al., 1997; Vaughn et al., 2000; Wang et al., 2006) and clinical trials of novel treatment approaches. Indeed, DENV RNA levels have been suggested to be a better virological endpoint in clinical trials of anti-viral therapies than NS1 concentrations (Tricou et al., 2010b). Second, the variability in diagnostic performance of in-house DENV RT-PCR assays in laboratories globally, and the rarity of publications describing their validation, suggests more transparent accounting of assay characteristics is desirable. Lastly, dengue is expanding its global footprint to include more developed countries where the capacity for molecular diagnostic methods is higher and where validated assay methods could be deployed. The aim of the current study was to develop and validate two internally controlled multiplex RT-PCR assays for serotype-specific detection and quantitation of DENV RNA in plasma.

2. Materials and methods

2.1. DEN virus isolates

Recent clinical isolates of DENV-1–4 from Vietnamese patients were used as reference viruses. The viruses used were DENV-1/VN/BID-V1792/2007 (Genotype I), DENV-2/VN/BID-V1002/2006 (Asian 1 genotype), DENV-3/VN/BID-V1329/2006 (Genotype 2) and DENV-4/VN/DENCO 33-188/2006.

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2.2. Equine Arteritis Virus (EAV) as an internal control

Equine Arteritis Virus (EAV), a positive-sense single-stranded RNA virus, was used as an internal control that was added to all specimens prior to RNA extraction. To generate working stocks of EAV, the virus was cultured on BHK-21 cells for 4–5 days. The culture supernatant was then harvested and the supernatant clarified by centrifugation at 17.7 × g for 5 min. The supernatant was then transferred to a separate sterile tube containing Bovine Serum Albumin (BSA) (20%, w/v in RPMI). Aliquots of supernatants containing EAV were prepared and frozen at −80°C. The amount of EAV to spike into clinical specimens was determined by titration experiments; the optimal final concentration was that which yielded Cp values in the range of 27–33.

2.3. Selection of primer and probe sequences

Primers for DENV-1–4 were selected on the basis of whole genome sequence alignments of Vietnamese and a global sample of DENV sequences. To identify the primers, 206 DENV-1 sequences, 198 DENV-2 sequences, 125 DENV-3 sequences, and 110 DENV-4 sequences (accession numbers available upon request) were aligned. Highly conserved sequences within each serotype were identified in the NS5 gene. Primer sequences were selected using Primer 3 software (Rozen and Skaletsky, 2000). Probe sequences for DENV-1, -3 and -4 were as described by Laue et al. (1999). The probe sequence for DENV-2 was selected on the basis of sequence alignments.

2.4. RNA extraction and real time one-step multiplex RT-PCR

The aim of this project was development of a multiplex RT-PCR that was (a) internally controlled, (b) could detect DENV-1–4 in a maximum of 2 assay tubes and (c) could be used for quantification. The primers and probes used in this study are described in Table 1. The optimum concentrations of primers and probes were deduced by titration experiments. Two multiplex reactions were established; the first contained primers/probes for DENV-2 and DENV-4 and the second contained primers/probes for DENV-1 and DENV-3. In all assay tubes a third primer/probe set was included—this was specific for the internal control target (EAV), standard and optimized aliquots of which had been spiked into each and every test specimen prior to RNA extraction.

Dengue viral RNA was isolated from 140 μL of plasma or 140 μL of DEN virus culture using the QIAamp Viral RNA Mini kit (QIA-GEN, Valencia, CA) according to the manufacturer's protocol. A real time one-step multiplex RT-PCR was optimized according to the manufacturer's instruction (RNA Master Hydrolysis Probes, Roche, Germany), with the exception of the activator volume, which was 1.4 μL per reaction. The DENV RNA was reverse transcribed at 61°C for 10 min, followed by 1 cycle of denaturation at 95°C for 2 min and amplification steps consisting of 45 cycles at 95°C for 15 s and 60°C for 30s. Amplification and detection was performed on a LightCycler 480II system (Roche, Germany).

2.5. Generating plasmid clones containing PCR amplimer for use as standard curve

To establish these plasmid clones for use as standard curves, the amplimers generated from each PCR were amplified and cloned into a pCR® 2.1-TOPO® cloning vector. Sequencing

of the cloned insert established the fidelity of the sequence. Plasmid DNA containing each amplimer sequence was purified, linearised by restriction enzyme digestion and then quantitated with the fluorescent nucleic acid stain picogreen. Two fold serial dilutions of this linearised plasmid DNA diluted in water were used as a standard curve in all assays.

2.6. Validation parameters

2.6.1. Specificity—A total of 100 plasma specimens were used in specificity testing. The panel consisted of 37 plasma samples from healthy volunteers and 63 plasma samples from patients with different infectious diseases including chronic HBV (n = 17) or HIV and HCV co-infection (n = 15), acute *Plasmodium falciparum* malaria (n = 15), acute *Enterovirus* 71 (n = 6) and acute pandemic H1N1 influenza (n = 8). Healthy plasma (10 replicates of each) was also spiked with Chikungunya virus and Japanese encephalitis virus.

2.6.2. Linearity—The linear range of each system was established and demonstrated by measuring 7–10 tenfold dilution concentration levels of DENV-1–4 (spiked into healthy donor plasma), and each dilution level was tested with 4 replicates. The distribution of each system was checked by Shapiro–Wilk test and the relationship between the observed values and true concentrations of analyte was examined through linear regression.

2.6.3. Limit of detection (LOD)—The LOD is the concentration at which one-step multiplex real time Dengue RT-PCR can detect a positive sample 95% of the time and is determined by Probit Analysis. Results obtained that were below the linear range (where the calibration curve was no longer valid), were considered as negative even if they were above the LOD.

2.6.4. Precision—The assessment of precision was performed at two concentra-tions (high and low) of DENV-1–4 spiked into healthy plasma, with 5 replicates at each concentration level. Precision was estimated by performing the RT-PCR assay twice per day over 5 days with each run separated by a minimum of 2 h. To examine repeatability for each serotype, a panel of 5 replicate plasma samples at each of two concentrations (high and low) of infectious DENV-1–4 spiked into healthy plasma was used (i.e. 10 samples per serotype); each panel was assayed (RNA extraction followed by RT-PCR) twice in a single day by the same technician using the same equipment and reagent lot numbers. There was a maximum of 2 h between assay runs. Intermediate precision was carried under the same conditions of the repeatability test except the time period was five different days. The distribution of each system was checked with Shapiro-Wilk test. To assess the equality of variances in repeatability test, Levene's test was used and intermediate precision was examined through one-way ANOVA.

2.7. Operational definitions

For testing of clinical specimens, a standard amount of internal control (EAV) is spiked into the sample. In all assay batches, external control samples include standard amounts of culture supernatants of DENV-1–4 and 1 negative control (molecular grade water). The samples and controls are processed in the exactly the same fashion as clinical specimens.

Measurement of viremia in patients is performed with the corresponding serotype standard curve (plasmid DNA).

The valid range and LOD of each serotype standard curve (plasmid DNA) was determined in the same manner as for infectious virus particles. Clinical specimens where the result is below the LOD are defined as “negative”. An assay is valid if all the external and internal controls yield results within a pre-defined range (within 10% of the mean value determined in the optimization experiments). An acceptable standard curve is that where the efficiency is >1.7, and the accuracy is <0.2. All analyses were performed with a polynomial equation.

3. Result

3.1. Limit of detection (LOD)-infectious virus particles

The sensitivity of each multiplex reaction was determined first using a 2-fold dilution series of cultured virus spiked into healthy donor plasma, with 60 replicates per dilution level. The LOD for each serotype was 1 PFU/mL for DENV-1, 1 PFU/mL for DENV-2, 0.5 $TCID_{50}$/mL for DENV-3 and 3 PFU/mL for DENV-4. The LOD findings are summarized in Table 2.

3.2. Limit of detection (LOD)-plasmid DNA

In a second assessment, the sensitivity of the method was determined against a dilution series of plasmid DNA in which the PCR target sequence had been cloned into a pCR® 2.1-TOPO® cloning vector and quantitated with the fluorescent nucleic acid stain picogreen. The LOD for each set of recombinant plasmids was 5 copies/reaction for DENV-1, 1 copy/reaction for DENV-2, 5 copies/reaction for DENV-3 and 10 copies/reaction for DENV-4 (Table 2).

3.3. Linearity

The extent of linearity in each system was determined by establishing titrations of infectious virus spiked into healthy plasma (4 replicates per dilution, 7–11 ten-fold dilutions per serotype). The results of linear regression are summarized in Table 3 including the parameters of the fitted models. The R^2 values attained for each assay ranged from 0.991 to 0.997 and were highly significant ($p < 0.001$). In addition, the lack of fit test for each system supported the linear regression models being used (Supplementary Table 1).

3.4. Specificity

To ascertain the specificity of each assay, a checkerboard style assessment was conducted to demonstrate that each primer/probe set assay was specific for its intended serotype, rather than another serotype(s). As expected, each assay amplified and detected only the intended serotype and there was no evidence cross-detection of other DENV serotypes (Fig. 1B). None of the plasma samples from healthy volunteers, patients with other infectious diseases whose clinical presentation might be similar to dengue, or are chronic infections that could be found in true dengue patients, or healthy plasma spiked with JEV or CHIV, yielded a positive result for any of the DENV-1–4 assays, indicating that the specificity of the assays was 100% in this panel of samples (data not shown).

3.5. Precision

The repeatability and intermediate precision of each assay was examined. Results of the repeatability assessment (Table 4) indicated there was no significant difference in results for each assay between assay runs. To examine intermediate precision, the same panel of high and low concentrations of infectious virus spiked into healthy plasma was used and assayed each panel twice per day on 5 consecutive days. Similarly, the intermediate precision was robust, with no significant differences of Cp value between days (Table 5).

3.6. Operational performance

This RT-PCR assay was used for laboratory investigations in two therapeutic intervention trials in dengue patients at the Hospital for Tropical Diseases, Ho Chi Minh City (ClinicalTrials.gov identifier: NCT01096576 and ISRCTN39575233) conducted during 2009–2011. The assay was conducted according to Good Clinical Laboratory Practices guidelines. Patients enrolled into these studies had dengue as determined by clinical signs and symptoms and positivity in a NS1 rapid test (NS1 STRIP, Biorad) and had <48 h of fever (NCT01096576) or <72 h of fever (ISRCTN39575233). In the 64 patients enrolled in NCT01096576 and 223 patients in ISRCTN39575233, 100% of enrolment plasma samples were positive by RT-PCR for one (and rarely, two) serotypes. The serotypes detected were DENV-1 (n = 168), DENV-2 (n = 78), DENV-3 (n = 28) and DENV-4 (n = 13). The coefficient of variation for the internal control virus EAV, spiked into each plasma specimen, was 5.53% for all samples (Fig. 1A). The coefficient of variation for the external control samples (standard aliquots of DEN virus cultures of known concentration) was 4.17% for DENV-1, 4.21% for DENV-2, 4.51% for DENV-3 and 4.58% for DENV-4. These data suggest the primers and probes being used were capable of detecting contemporary DEN viruses circulating in southern Viet Nam and the low %CV of the internal and external controls suggests high reproducibility in assay performance.

4. Discussion

A rapid and internally controlled DENV RT-PCR multiplex assay was developed and validated that can identify four DEN virus serotypes in two reaction mixtures. The validation parameters described here were in accordance with procedural and statistical methods as recommended in the Validation of Analytical Procedures: Text and Methodology, ICH guidelines (ICH, 1995). The strengths of this method are the ability to detect all four DENV serotypes currently in circulation in Viet Nam, the ability to perform within sample validation of extraction, cDNA synthesis and PCR steps via use of an internal control and the high sensitivity and specificity. The specificity of the assay was determined against related viruses (JEV and HCV), but also in clinical specimens from patients with infectious diseases common in Viet Nam. The advantages over nested RT-PCR approaches that rely on gel electrophoresis are clear—the method described here is faster, quantitative, less prone to contamination and can be validated. These characteristics make it attractive for use in clinical research, where the viremia level in plasma can be used as an endpoint in studies of pathogenesis and therapeutic intervention trials (Dung et al., 2010; Tricou et al., 2010a). Other RT-PCR methods for detection of DEN viruses have been described and share

some of the features of the method described here in terms of a real-time multiplex (Chien et al., 2006; Naze et al., 2009; Sadon et al., 2008).

Like all positive-stranded RNA viruses, DEN viruses accumulate mutations during replication in their human and mosquito hosts. As a consequence, within each DEN virus serotype there are phylogenetically distinct clades, or genotypes. The maximum nucleotide sequence diversity between viruses in the same serotype is ~8% (Vu et al., 2010). A consequence is that primers and probes used in RT-PCR assays can occasionally be mismatched to sequences in the virus. In order to limit the likelihood of this, the targeted amplification sequence was in the NS5 gene, a region of very high sequence conservation within each serotype, and to a lesser extent, also between serotypes. Despite the high level of sequence conservation between serotypes in this region of the virus genome, nonetheless suitable primer/probe sequences that discriminated between serotypes were identified with no cross-reactivity with JEV.

Despite the global burden of dengue and the number of countries affected, there are surprisingly few descriptions of validated DEN virus specific molecular diagnostic assays in the literature. A further challenge to dengue molecular diagnostics is the absence of formal and ongoing external quality assurance programs that can assist diagnostic laboratories for verifying and maintaining robust diagnostic tests. A recently circulated evaluation panel for DEN virus RT-PCR was academic in origin but nonetheless highlighted variability in sensitivity and even specificity between assays and laboratories (Domingo et al., 2010). Given the increasing penetration of molecular diagnostic capacity in many dengue endemic countries, the establishment of a formal EQA program for dengue molecular diagnostics is a worthwhile goal.

Molecular diagnosis is not essential for laboratory confirmation of dengue. Serological and NS1 antigen detection methods are also robust (Guzman et al., 2010). However, serological methods are not sensitive in the first few days of fever. Diagnosis by NS1 detection can be achieved in the first few days of fever, but not in all cases and there is evidence of a serotype dependent feature to NS1 detection (Hang et al., 2009; Tricou et al., 2010b). Molecular methods of DEN virus RNA detection in plasma/serum, when conducted in a stringent and controlled diagnostic laboratory setting, offer the most reliable method of very early diagnosis and this will be critical for the clinical development, if not the deployment, of anti-viral therapeutic interventions in dengue. Moreover, there is a body of literature that suggests early DEN virus viremia levels have prognostic significance. Thus, in the future it might be feasible to identify patients at higher risk of developing severe complications on the basis of their viremia level. This might lead to different triage and management for “high risk” patients. To reach this goal will require more clinical research to develop a clinical useful algorithm that includes early viremia level as a variable (Tanner et al., 2008), but also other variables recognized as being contributors to outcomes, e.g. age, gender, immune status with respect to previous DEN virus infections (Anders et al., 2011).

In summary an internally controlled, DEN virus specific RT-PCR assay has been developed, validated and characterized operationally and this and assays like it are important for studies of pathogenesis, vaccine and drug development. In the future, it is likely that molecular

diagnostic assays will become more widely used for the routine diagnosis of dengue in endemic countries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

DENV	dengue virus
RT	reverse transcription
NS5	nonstructural protein 5
LOD	limit of detection
F	forward primer
R	reverse primer

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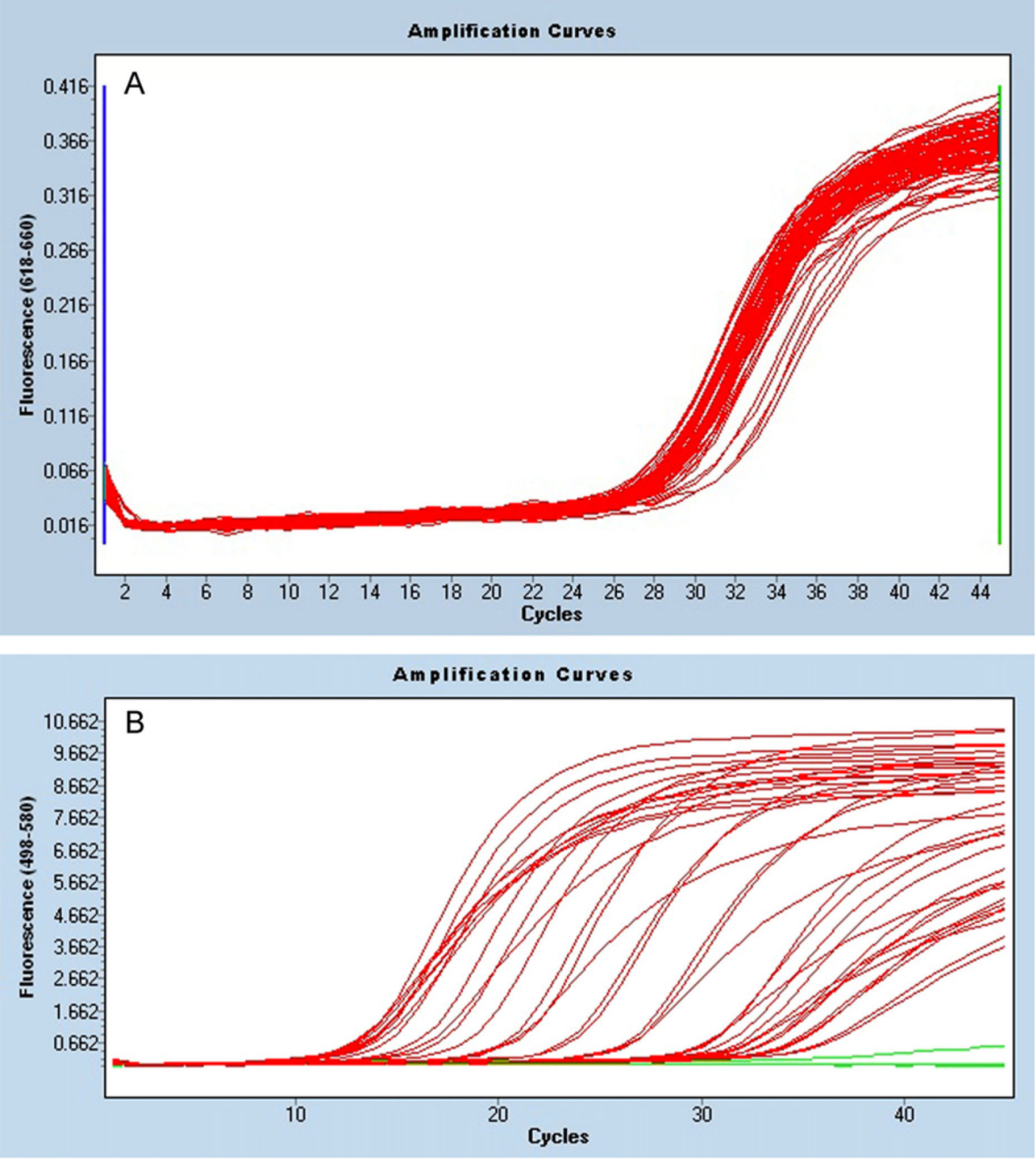

Fig. 1.

(A) The highly reproducible signal derived from the EAV internal control in 46 plasma samples and (B) the specific signal for DENV-1 in 46 plasma samples.

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Table 1

Primers/probes and optimal concentrations.

Serotype	Primer/probe sequences (5′–3′)	Position	Final conc. (μM/reaction)
DENV-1-F	ATCCATGCCCAYCACCAAT	9865–9883	1
DENV-1-R	TGTGGGTTTTGTCCTCCATC	9945–9964	1
DENV-1-Probe	FAM-TCAGTGTGGAATAGGGTTTGGATAGAGGAA-BHQ1	9907–9936	0.14
DENV-2-F	TCCATACACGCCAAACATGAA	9859–9879	1
DENV-2-R	GGGATTTCCTCCCATGATTCC	9963–9983	1
DENV-2-Probe	FAM-AGGGTGTGGATTCGAGAAAACCCATGG-BHQ1	9916–9942	0.14
DENV-3-F	TTTCTGCTCCCACCACTTTC	9591–9610	1
DENV-3-R	CCATCCYGCTCCTTGAGA	9691–9708	1
DENV-3-Probe	Cyan500-AAGAAAGTTGGTAGTTCCCTGCAGACCCCA-BHQ1	9633–9662	0.14
DENV-4-F	GYGTGGTGAAGCCYCTRGAT	9587–9607	1
DENV-4-R	AGTGARCGGCCATCCTTCAT	9744–9764	1
DENV-4-Probe	Cyan500-ACTTCCCTCCTCTTYTTGAACGACATGGGA-BHQ1	9490–9519	0.14
EAV-F	CATCTCTTGCTTTGCTCCTTAG	1847–1868	0.2
EAV-R	AGCCGCACCTTCACATTG	1980–1997	0.2
EAV-Probe	Cy5-CGCGCTCGCTGTCAGAACAACATTATTGCCCACAGCGCG-BHQ3	1926–1964	0.08

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Table 2

The LOD of each assay against infectious virus (spiked into healthy donor plasma) and plasmid DNA.

	DENV-1	DENV-2	DENV-3	DENV-4
Infectious virus[a]	1	1	0.5	3
Copy number of "DNA standard" per RT-PCR[b]	5	1	5	10

[a] For DENV-1, -2, -4, the unit is plaque forming units per mL, for DENV-3 the unit is $TCID_{50}$ units.

[b] "DNA standard" is cloned PCR amplimer in a linearised plasmid.

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Table 3

Result ofsimple linear regression of linearity data for each DENV serotype.

	Estimate	Standard error	*p*-Value	R^2	Linear range
DENV-1				0.994	7.6E+4–0.76 (PFU/mL)
Intercept	31.155	0.15	<0.001		
Slope	−3.187	0.051	<0.001		
DENV-2				0.997	1.0E+5–1 (PFU/mL)
Intercept	33.33	0.097	<0.001		
Slope	−2.976	0.032	<0.001		
DENV-3				0.991	1.0E+4–0.1 ($TCID_{50}$/mL)
Intercept	29.368	0.161	<0.001		
Slope	−3.499	0.071	<0.001		
DENV-4				0.994	2.0E+4-2 (PFU/mL)
Intercept	33.82	0.172	<0.001		
Slope	−3.538	0.063	<0.001		

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Table 4

Repeatability test for each serotype.

Conc.	System	Mean of Cp	Std. dev	*p*-Value[a]
7.6E+04PFU/mL	DENV-1	18.586	0.050	0.244
7.6PFU/mL		31.590	0.034	0.716
1.0E+5 PFU/mL	DENV-2	18.496	0.029	0.844
10PFU/mL		30.490	0.024	0.999
1.0E+03 $TCID_{50}$/mL	DENV-3	18.509	0.038	0.429
1$TCID_{50}$/mL		29.537	0.036	0.347
2.0E+4 PFU/mL	DENV-4	17.752	0.030	0.832
20 PFU/mL		30.525	0.039	0.796

[a] *p*-Value of the Levene's test.

Table 5

Intermediate precision test of each serotype.

Conc.	System	Sum of squares	Degreed of freedom	Mean squares	*F*-ratio	*p*-Value[a]
7.6E+04 PFU/mL	DENV-1	0.007	4	0.002	0.800	0.529
7.6PFU/mL		0.003	4	0.001	0.420	0.790
1.0E+5 PFU/mL	DENV-2	0.002	4	0.001	0.630	0.642
10 PFU/mL		0.008	4	0.002	1.500	0.219
1.0E+03$TCID_{50}$/mL	DENV-3	0.006	4	0.002	0.56	0.689
1$TCID_{50}$/mL		0.003	4	0.001	0.35	0.844
2.0E+4 PFU/mL	DENV-4	0.001	4	0.000	0.320	0.863
20 PFU/mL		0.005	4	0.001	0.710	0.589

[a] *p*-Value of F test.