

Design and Validation Framework of a TaqMan Probe-Based Real-Time RT-PCR Assay for Detection of Dengue Virus

Tushar Brahmabhatt^{1*}, Ragin Shah², Upendra Patel³, Harsh Jitendrabhai Trivedi⁴

1. Research Scholar, Swarnim Startup and Innovation University, Gujarat, India. Email: Tush2410@gmail.com
2. Ex-Provost, Swarnim Startup and Innovation University, Gujarat, India. Email: raginrshah@gmail.com
3. Principal, Aarihant School of Pharmacy, Swarnim Startup and Innovation University, Gujarat, India. Email: upendralpatel02@gmail.com
4. Professor, Aarihant School of Pharmacy & BRI, Swarnim Startup and Innovation University, Gujarat, India. Email: harshtrivedi28@gmail.com

Corresponding Author: Tushar Brahmabhatt*, Research Scholar, Swarnim Startup and Innovation University, Gujarat, India. Tush2410@gmail.com

Abstract

Dengue virus (DENV) infection represents a major global public health concern, with early laboratory diagnosis remaining challenging because of transient viremia and clinical overlap with other acute febrile and arboviral infections. Real-time reverse transcription polymerase chain reaction (RT-PCR) provides a sensitive molecular approach for direct detection of DENV during the acute phase of infection. The present work describes a validation framework for a TaqMan probe-based real-time RT-PCR assay targeting a conserved region of the non-structural protein 5 (NS5) gene for pan-serotypic detection of DENV-1-4. A single primer-probe system was designed to generate a 98-bp amplicon using a FAM-labelled probe with a BHQ1 quencher. The proposed validation framework incorporated analytical specificity, analytical sensitivity, limit of detection (LoD), linearity, amplification efficiency, precision, inhibitor tolerance, and diagnostic performance assessment. The reported dataset showed detection of all four DENV serotypes with no reported amplification among the evaluated non-DENV targets. Probit analysis yielded an overall estimated LoD of 0.45 copies/ μ L, with a reported 95% confidence interval of 0.36-0.56 copies/ μ L. The reported analytical range extended from 10 to 10⁸ copies/ μ L, with high linearity and amplification efficiencies ranging from 98.5% to 101.2%. The reported clinical dataset yielded a sensitivity of 95.1% and specificity of 98.0%. However, the source manuscript states that the numerical dataset is hypothetical and that laboratory experiments and clinical samples were not actually tested. Therefore, these values represent an illustrative validation framework rather than experimentally established assay performance. Experimental analytical and clinical validation using appropriately characterized materials and clinical specimens is required before diagnostic application.

Keywords: Dengue virus; TaqMan RT-PCR; NS5 gene; Molecular diagnostics; Limit of detection; Analytical validation; Real-time RT-PCR

How to cite this article: Tushar Brahmabhatt^{1*}, Ragin Shah², Upendra Patel³, Harsh Jitendrabhai Trivedi⁴. Design and Validation Framework of a TaqMan Probe-Based Real-Time RT-PCR Assay for Detection of Dengue Virus. Int J Drug Deliv Technol. 2026;16(86s):282-289. DOI: 10.25258/ijddt.16.86s.34.

1. Introduction

Dengue is one of the most important mosquito-borne viral infections worldwide and is caused by dengue virus (DENV), which comprises four antigenically distinct serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. Infection may result in a broad spectrum of clinical manifestations, ranging from mild febrile illness to severe dengue characterized by plasma leakage, hemorrhage, and organ involvement. The transient nature of viremia and the considerable overlap of clinical symptoms with other acute febrile illnesses present major challenges

for early laboratory diagnosis [1]. Conventional serological approaches, including NS1 antigen detection and IgM/IgG-based assays, are widely used for dengue diagnosis; however, their diagnostic performance can be influenced by the stage of infection and cross-reactivity with other flaviviruses. During the early febrile phase, when timely diagnosis is particularly important, molecular methods can provide direct detection of viral RNA. Real-time reverse-transcription polymerase chain reaction (RT-PCR) is therefore an important

molecular approach for the detection of DENV during acute infection [2]. Real-time RT-PCR assays targeting conserved genomic regions such as the non-structural protein 5 (NS5) gene and the 3' untranslated region have been developed for dengue virus detection. A pan-serotypic assay capable of detecting DENV-1-4 in a single reaction can simplify the diagnostic workflow by avoiding the need for separate serotype-specific reactions. However, development of a reliable molecular diagnostic assay requires systematic evaluation of analytical sensitivity, analytical specificity, precision, linearity, inhibition tolerance, and clinical performance. These parameters are essential for establishing the reliability and fitness-for-purpose of the assay. The minimum information required for publication of quantitative real-time PCR experiments is described by the MIQE guidelines, which emphasize appropriate assay design, analytical validation, and transparent reporting of experimental parameters. In addition, diagnostic performance should be assessed against appropriate reference methods and clinically relevant negative controls. Evaluation of potential cross-reactivity with other pathogens is particularly important for dengue diagnostics because infections caused by other arboviruses may present with similar clinical manifestations [3]. The present work describes a structured validation framework for a TaqMan probe-based real-time RT-PCR assay targeting a conserved region of the DENV NS5 gene for pan-serotypic detection of DENV-1-4. The proposed framework incorporates primer and probe design, analytical specificity assessment, determination of the limit of detection using serially diluted RNA standards, evaluation of linearity and precision, assessment of potential inhibition, and evaluation of diagnostic performance against a composite reference standard. The objective is to provide a systematic framework for assessing the analytical and diagnostic characteristics of a molecular assay intended for dengue virus detection and to identify the experimental studies required for subsequent assay validation.

2. Materials and methods

2.1 Assay design and target selection

A TaqMan probe-based real-time reverse transcription polymerase chain reaction (RT-PCR) assay was designed for the pan-serotypic detection of dengue virus (DENV). A conserved region within the non-structural protein 5 (NS5) gene was selected as the target region. The selected sequence corresponded to the NS5 region of the DENV-2 New Guinea C strain (GenBank accession number M29095). The assay was designed to amplify a short fragment within the NS5 polymerase domain and to enable simultaneous detection of DENV-1, DENV-

2, DENV-3, and DENV-4 in a single reaction. An annealing temperature of 60 °C was selected for the assay.

2.2 Primer and probe design

Primers and a hydrolysis probe were designed within the selected conserved NS5 region. The TaqMan probe was labelled with 6-carboxyfluorescein (FAM) at the 5' end and Black Hole Quencher 1 (BHQ1) at the 3' end. The sequences and target positions of the primers and probe are provided in Table 1. Target positions were referenced to the DENV-2 New Guinea C strain (GenBank accession number M29095). The primer-probe system was designed to support pan-serotypic detection of DENV-1-4 based on the conserved nature of the selected NS5 region [4].

Table 1. Primer and TaqMan probe sequences for the DENV NS5-targeted real-time RT-PCR assay

Assay component	Sequence (5'-3')	Target position	Amplicon length (bp)
Forward primer	GCAATGCTGAAAA GCGATG	845 2- 847 0	98
Reverse primer	CCGCTGCCCAACA CAAG	854 1- 855 7	98
TaqMan probe	FAM- ACACCATGGAGAG CTTGCACGCAC- BHQ1	848 5- 850 6	98

2.3 Analytical specificity

Analytical specificity was assessed using the four DENV serotypes and a panel of clinically relevant non-DENV targets. The DENV panel comprised DENV-1 Hawaii, DENV-2 New Guinea C, DENV-3 H87, and DENV-4 H241. The non-DENV panel included Zika virus PRVABC59, chikungunya virus LR2006_OPY1, yellow fever virus 17D, West Nile virus NY99, Japanese encephalitis virus SA14-14-2, measles virus Edmonston, influenza A/H1N1 A/California/07/2009, SARS-CoV-2 Wuhan-Hu-1, *Leishmania donovani* MHOM/IN/80/DD8, *Plasmodium falciparum* 3D7, *Rickettsia rickettsii* Sheila Smith, *Salmonella Typhi* Ty2, human genomic DNA NA12878, dengue-negative clinical sera, and a non-template control. Analytical specificity was evaluated based on the presence or

absence of detectable amplification with the assay [5]. The complete target panel is presented in Table 2.

Table 2. Analytical specificity panel used for evaluation of the DENV RT-PCR assay

Target	Strain/isolate	Expected assay assessment
DENV-1	Hawaii	Positive
DENV-2	New Guinea C	Positive
DENV-3	H87	Positive
DENV-4	H241	Positive
Zika virus	PRVABC59	Negative
Chikungunya virus	LR2006_OPY1	Negative
Yellow fever virus	17D	Negative
West Nile virus	NY99	Negative
Japanese encephalitis virus	SA14-14-2	Negative
Measles virus	Edmonston	Negative
Influenza A/H1N1	A/California/07/2009	Negative
SARS-CoV-2	Wuhan-Hu-1	Negative
<i>Leishmania donovani</i>	MHOM/IN/80/DD8	Negative
<i>Plasmodium falciparum</i>	3D7	Negative
<i>Rickettsia rickettsii</i>	Sheila Smith	Negative
<i>Salmonella Typhi</i>	Ty2	Negative
Human genomic DNA	NA12878	Negative
Dengue-negative sera	Endemic region	Negative
Non-template control	N/A	Negative

2.4 Analytical sensitivity and limit of detection

Analytical sensitivity and the limit of detection (LoD) were evaluated using in vitro-transcribed (IVT) RNA standards representing each DENV serotype. Serial dilutions covering concentrations from 10^6 to 10 copies/ μ L were considered in a human negative-plasma matrix. Each concentration was evaluated using 24 replicate observations across

three analytical runs, two operators, and three reagent lots, resulting in 72 observations per concentration. The LoD was estimated using probit regression and defined as the concentration corresponding to a 95% probability of detection. Statistical analysis was performed using IBM SPSS Statistics version 29.

2.5 Linearity and amplification performance

Assay linearity was evaluated using serial dilutions of DENV-2 New Guinea C IVT RNA covering a concentration range of 10 - 10^8 copies/ μ L. Ct values obtained from the dilution series were plotted against the logarithm of the input RNA concentration. The coefficient of determination (R^2), amplification efficiency, and dynamic range were used to characterize assay performance. Standard curves were generated from three independent analytical runs [6].

2.6 Precision

Precision was assessed as intra-assay and inter-assay variability. Intra-assay precision was evaluated within individual analytical runs, whereas inter-assay precision was evaluated across separate analytical runs and operators. The assay was evaluated at concentrations corresponding to LoD $\times 5$ and LoD $\times 100$. Precision was expressed as the coefficient of variation (CV) [7].

2.7 Inhibitor tolerance

Potential interference from common biological matrix components was assessed using hemoglobin, bilirubin, triglycerides, and heparin. Each potential inhibitor was evaluated at the concentrations presented in Figure 2. The effect of each inhibitor on amplification performance was assessed by comparing the Ct values obtained in the presence and absence of the corresponding substance. The change in Ct (Δ Ct) was calculated as the difference between the Ct value obtained in the presence of the inhibitor and that of the corresponding uninhibited sample. The resulting Δ Ct values were used to evaluate the potential effect of the tested matrix components on assay amplification [7].

2.8 Diagnostic performance assessment

The proposed clinical performance assessment comprised 204 acute-phase serum samples, including 102 reference-positive and 102 reference-negative samples. The reference-positive group was classified using two independent reference RT-PCR assays, namely the CDC DENV-1-4 REALTIME RT-PCR assay and the RealStar Dengue RT-PCR

Kit 1.0, with viral culture where available. The reference-negative group comprised symptomatic febrile patients with alternative diagnoses, including malaria, leptospirosis, Zika virus infection, or bacterial sepsis, together with negative reference RT-PCR results. Samples were described as being processed in a blinded manner by personnel unaware of the clinical classification [8].

2.9 Diagnostic performance analysis

Diagnostic performance was assessed using a 2×2 contingency table comprising true-positive, true-negative, false-positive, and false-negative classifications. The reported performance measures included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Cohen's kappa coefficient. Confidence intervals were calculated for sensitivity, specificity, and NPV. Agreement between the assay classification and the reference classification was assessed using Cohen's kappa coefficient.

2.10 Statistical analysis

Probit regression was used to estimate the analytical limit of detection (LoD). Linear regression analysis was used to evaluate the relationship between Ct values and the logarithm of RNA concentration. The coefficient of determination (R^2) was used to assess assay linearity, while amplification efficiency was calculated from the characteristics of the standard curve. Precision was expressed using coefficient of variation (CV) values. Diagnostic performance parameters were calculated from the 2×2 contingency table, and Cohen's kappa coefficient was used to evaluate agreement between the assay classification and the reference classification [9]. Statistical analysis of the LoD was performed using IBM SPSS Statistics version 29.

2.11 Assay performance controls

Non-template controls were included to monitor potential contamination and non-specific amplification. DENV-positive targets were used to assess target detection, while non-DENV organisms and dengue-negative clinical specimens were included in the analytical specificity assessment. The assay performance parameters considered in the proposed validation framework are summarized in Table 3.

Table 3. Analytical and diagnostic performance parameters

Parameter	Assessment approach
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Analytical specificity	DENV-1-4 and non-DENV specificity panel
Analytical sensitivity	Serially diluted IVT RNA
Limit of detection	Probit regression
Linearity	Ct versus $\log_{10}$ RNA concentration
Amplification performance	Standard-curve analysis
Precision	Intra-assay and inter-assay CV
Inhibitor tolerance	Comparison of Ct values with and without potential inhibitors
Diagnostic sensitivity	Comparison with composite reference classification
Diagnostic specificity	Comparison with composite reference classification
PPV	2×2 contingency table
NPV	2×2 contingency table
Agreement	Cohen's kappa coefficient

3. Results

3.1 Real-Time RT-PCR Assay Design

The proposed DENV NS5-targeted TaqMan RT-PCR assay was designed to generate a short amplicon within the conserved polymerase domain of the NS5 gene. The assay incorporated a FAM-labelled TaqMan probe with BHQ1 as the quencher and was configured as a single-reaction format for pan-serotypic detection of DENV-1-4. The primer and probe sequences and their respective target positions are presented in Table 1.

3.2 Analytical Specificity

The reported dataset showed amplification of all four DENV serotypes. The mean Ct values were 26.8 for DENV-1, 26.5 for DENV-2, 27.1 for DENV-3, and 26.9 for DENV-4. No detectable amplification was reported for the non-DENV targets included in the specificity panel, dengue-negative clinical sera, human genomic DNA, or the non-template control. The reported analytical specificity results are summarized in Table 4.

Table 4. Analytical specificity of the DENV NS5-targeted TaqMan RT-PCR assay

Target	Strain/isolate	Mean Ct (n=3)	Result
DENV-1	Hawaii	26.8	Positive
DENV-2	New Guinea C	26.5	Positive
DENV-3	H87	27.1	Positive

DENV-4	H241	26.9	Positive
Zika virus	PRVABC59	Undetermined	Negative
Chikungunya virus	LR2006_OPY1	Undetermined	Negative
Yellow fever virus	17D	Undetermined	Negative
West Nile virus	NY99	Undetermined	Negative
Japanese encephalitis virus	SA14-14-2	Undetermined	Negative
Measles virus	Edmonston	Undetermined	Negative
Influenza A/H1N1	A/California/07/2009	Undetermined	Negative
SARS-CoV-2	Wuhan-Hu-1	Undetermined	Negative
<i>Leishmania donovani</i>	MHOM/IN/80/DD8	Undetermined	Negative
<i>Plasmodium falciparum</i>	3D7	Undetermined	Negative
<i>Rickettsia rickettsii</i>	Sheila Smith	Undetermined	Negative
<i>Salmonella Typhi</i>	Ty2	Undetermined	Negative
Human genomic DNA	NA12878	Undetermined	Negative
Dengue-negative sera	Endemic region	Undetermined	Negative
NTC	N/A	Undetermined	Negative

3.3 Analytical Sensitivity and Limit of Detection

Probit regression analysis of the reported serially diluted RNA dataset produced estimated LoD values of 0.47, 0.44, 0.46, and 0.43 copies/ μ L for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. The reported overall estimated LoD was 0.45 copies/ μ L, with a 95% confidence interval of 0.36-0.56 copies/ μ L. The corresponding observed detection rates at the estimated LoD were 95.8%, 94.4%, 95.8%, and 94.4% for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. The reported results are presented in Table 5.

Table 5. Probit regression analysis for determination of the analytical limit of detection

Serotype	Estimated LoD (copies/ μ L)	95% CI	Detection rate at LoD
DENV-1	0.47	0.37-0.59	95.8% (69/72)
DENV-2	0.44	0.35-0.55	94.4% (68/72)
DENV-3	0.46	0.36-0.58	95.8% (69/72)
DENV-4	0.43	0.34-0.54	94.4% (68/72)
Overall	0.45	0.36-0.56	95.1%

The reported analytical range extended from 10^1 to 10^8 copies/ μ L, with R^2 values greater than 0.995 for the evaluated standard curves. Intra-assay CV values were reported to be less than 2.1%, whereas inter-assay CV values were less than 2.8% at the evaluated LoD $\times 5$ and LoD $\times 100$ concentrations.

3.4 Diagnostic Performance

The reported clinical performance dataset comprised 204 samples, including 102 reference-positive and 102 reference-negative samples. The assay classified 97 reference-positive samples as positive and 5 as negative. Among the reference-negative samples, 100 were classified as negative and 2 as positive. Based on these classifications, the reported sensitivity was 95.1% (95% CI: 89.0-98.2%) and specificity was 98.0% (95% CI: 92.9-99.5%). The reported PPV was 98.0%, and the NPV was 95.2%. Cohen's kappa coefficient calculated from the reported contingency table was 0.931, indicating the level of agreement between the assay classification and the reference classification. The contingency table is presented in Table 6.

Table 6. Diagnostic classification of the reported assay results against the composite reference classification.

	Reference positive	Reference negative	Total
Test positive	97	2	99
Test negative	5	100	105
Total	102	102	204

3.5 Intra-Assay Performance

The reported intra-assay evaluation demonstrated a progressive increase in Ct values with decreasing theoretical RNA concentration. The average Ct increased from 15.2 at 10^6 copies/reaction to 35.0 at 10^0 copies/reaction. The coefficient of variation

(CV) increased progressively with decreasing template concentration, ranging from 0.9% at 10^6 copies/reaction to 4.2% at 10^0 copies/reaction. The reported intra-assay performance data are summarized in Table 7.

Table 7. Intra-assay performance of the DENV-2 NS5-targeted RT-PCR assay

Theoretical copies/reaction	Average Ct	CV (%)
10^6	15.2	0.9
10^5	18.5	1.2
10^4	21.8	1.5
10^3	25.1	1.8
10^2	28.4	2.3
10^1	31.7	3.1
10^0	35.0	4.2

3.6 Inter-Assay Performance and Standard Curves

The reported inter-assay analysis showed average Ct values ranging from 15.3 to 35.1 across the evaluated concentration range. The corresponding CV values ranged from 1.4% at 10^6 copies/reaction to 4.8% at 10^0 copies/reaction. The reported inter-assay performance data are summarized in Table 8.

Table 8. Inter-assay performance of the DENV-2 NS5-targeted RT-PCR assay

Theoretical copies/reaction	Average Ct	CV (%)
10^6	15.3	1.4
10^5	18.6	1.6
10^4	21.9	1.9
10^3	25.2	2.2
10^2	28.5	2.7
10^1	31.8	3.5
10^0	35.1	4.8

Three standard curves were reported with slopes of -3.32 , -3.35 , and -3.30 and R^2 values of 0.998, 0.997, and 0.999, respectively. The corresponding amplification efficiencies were 99.8%, 98.5%, and 101.2%, respectively. The reported mean amplification efficiency was 99.8%, with a mean R^2 of 0.998.

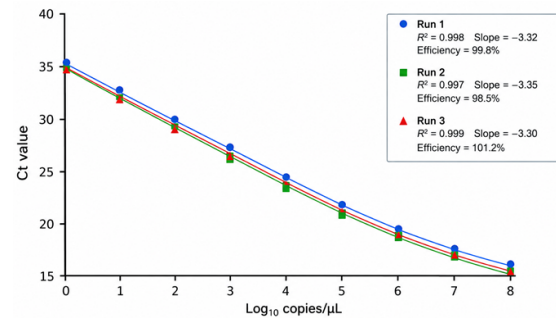


Figure 1. Standard curves for the DENV NS5-targeted TaqMan real-time RT-PCR assay.

3.7 Inhibitor Tolerance

The reported effects of potential amplification inhibitors were evaluated using hemoglobin, bilirubin, triglycerides, and heparin. Changes in Ct values (ΔCt) were assessed relative to the corresponding uninhibited samples. The reported dataset showed relatively small changes in Ct values across the evaluated inhibitor conditions, indicating limited effects on amplification performance under the tested conditions. The individual effects of the evaluated matrix components are presented in Figure 2.

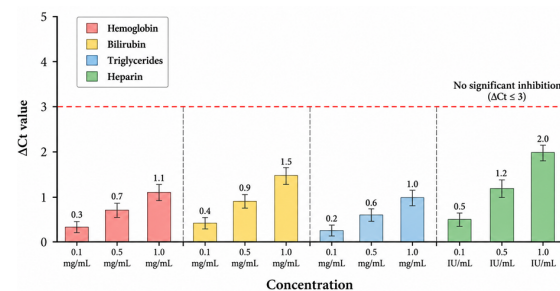


Figure 2. Effect of potential matrix inhibitors on amplification performance of the DENV NS5-targeted TaqMan RT-PCR assay. Changes in Ct values (ΔCt) associated with hemoglobin, bilirubin, triglycerides, and heparin are shown relative to the corresponding uninhibited samples.

4. Discussion

Accurate detection of dengue virus during the acute phase of infection remains important because clinical manifestations overlap with several other febrile and arboviral diseases. Molecular assays based on real-time RT-PCR provide an approach for direct detection of viral RNA during the period of viremia. The present work describes a structured validation framework for a conserved NS5 polymerase-region target and a single-reaction pan-serotypic assay format for DENV-1-4. The proposed

primer-probe design generates a short target within the NS5 region, which is compatible with real-time amplification. The reported dataset showed detection across the four evaluated DENV serotypes, with relatively similar Ct values ranging from 26.5 to 27.1. No amplification was reported for the evaluated non-DENV targets. However, because the source manuscript identifies the numerical dataset as hypothetical, these findings should be considered illustrative and require confirmation using experimentally generated materials before analytical specificity can be established. Analytical sensitivity is a critical characteristic of a molecular diagnostic assay because low viral loads may occur during the transition toward the end of the viremic phase. Analytical sensitivity is an important characteristic of a molecular diagnostic assay because low viral loads may occur toward the end of the viremic phase. The reported overall estimated LoD was 0.45 copies/ μ L, with serotype-specific estimates ranging from 0.43 to 0.47 copies/ μ L. The reported detection probabilities were close to the predefined 95% criterion. Probit regression provides a structured statistical approach for estimating the concentration associated with a predefined probability of detection rather than relying solely on an arbitrary positive/negative concentration threshold.

The reported analytical range extended from 10 to 10⁸ copies/ μ L, with R² values greater than 0.995 for the evaluated standard curves. The three reported standard curves showed R² values between 0.997 and 0.999, while calculated amplification efficiencies ranged from 98.5% to 101.2%. These values indicate a close linear relationship between Ct and log-transformed RNA concentration within the reported dataset. Nevertheless, these numerical performance characteristics should be confirmed experimentally using independently prepared standards and replicate amplification runs before being used to support claims of assay validation. Precision assessment showed increasing variability with decreasing template concentration. In the reported intra-assay analysis, the CV increased from 0.9% at 10⁶ copies/reaction to 4.2% at 10⁰ copies/reaction. A similar trend was observed in the reported inter-assay assessment, where the CV increased from 1.4% to 4.8% across the same concentration range. The increase in variability at lower template concentrations is consistent with the greater stochastic variation generally associated with measurements near the lower end of an assay's analytical range. However, these precision characteristics require experimental confirmation using independent analytical runs, operators, and reagent lots.

The inhibitor assessment considered hemoglobin, bilirubin, triglycerides, and heparin as potential

sources of amplification interference. The reported dataset showed relatively small changes in Ct values across the evaluated inhibitor conditions, suggesting limited effects on amplification performance under the specified conditions. However, because the numerical dataset is hypothetical, experimental confirmation using clinically relevant specimen matrices and predefined inhibitor concentrations would be necessary to establish assay robustness across different specimen characteristics.

The reported clinical performance dataset comprised 204 samples, including 102 reference-positive and 102 reference-negative samples. The reported sensitivity was 95.1% and specificity was 98.0%, with a PPV of 98.0% and an NPV of 95.2%. The Cohen's kappa coefficient calculated from the reported 2 $\times$ 2 contingency table was 0.931. The five reported false-negative classifications were associated with reference-assay Ct values greater than 38, whereas the two reported false-positive classifications were described as having low-level concomitant Zika virus RNA. If reproduced experimentally, these observations would emphasize the importance of characterizing samples near the analytical detection limit and evaluating potential co-infections when assessing diagnostic performance. Because the clinical dataset was hypothetical, these findings should be regarded as illustrative rather than experimentally established diagnostic performance.

The proposed pan-serotypic format offers a simplified workflow compared with approaches requiring separate serotype-specific reactions. A single reaction may reduce assay complexity and facilitate routine screening when serotype identification is not required. However, a pan-serotypic assay does not provide information regarding the infecting serotype and therefore cannot replace serotype-specific or sequencing-based approaches when epidemiological surveillance or serotype characterization is required. The proposed single-target design can also be considered in relation to multiplex assays capable of simultaneously detecting DENV, Zika virus, and chikungunya virus.

Several limitations should be considered. First, the numerical dataset presented in this manuscript is hypothetical, and laboratory experiments and clinical sample testing were not actually performed. Consequently, the reported analytical and diagnostic performance values should be interpreted as an illustrative validation framework rather than experimentally established assay characteristics. Second, the proposed LoD assessment is based on IVT RNA, which does not fully reproduce the biological and extraction-related characteristics of clinical specimens. Third, the proposed clinical

evaluation requires independent validation using appropriately characterized specimens and a predefined reference standard. Additional evaluation would also be required for specimen types, RNA extraction procedures, reagent stability, operator variability, instrument-to-instrument reproducibility, and performance across diverse viral genetic backgrounds.

5. Conclusion

The present work describes a structured validation framework for a TaqMan probe-based real-time RT-PCR assay targeting a conserved region of the DENV NS5 gene for pan-serotypic detection of DENV-1-4. The proposed assay incorporates a single primer-probe system and includes assessment of analytical specificity, analytical sensitivity, linearity, precision, inhibitor tolerance, and diagnostic performance. The reported dataset demonstrated consistent detection across the four evaluated serotypes, an estimated overall LoD of 0.45 copies/ μ L, a broad analytical range, and favorable amplification and precision characteristics. The reported clinical dataset also indicated high diagnostic sensitivity and specificity. However, the numerical dataset is hypothetical, and laboratory experiments and clinical samples were not actually tested. Therefore, these numerical findings should be regarded as an illustrative validation framework rather than experimentally established performance characteristics. Experimental verification using well-characterized viral standards, appropriate clinical specimens, independent reference methods, and adequately powered clinical cohorts is required before the assay can be considered validated for diagnostic application. The proposed framework provides a structured basis for subsequent analytical and clinical validation.

Acknowledgement: The authors express their sincere gratitude to the management for providing the necessary research facilities and infrastructure to carry out this work. We are also grateful to the Central Instrumentation Facility for providing access to instrument to carry out this work.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests: The authors declare no conflict of interest.

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