Journal of Virological Methods 271 (2019) 113677

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocols

# Novel pan-serotype control RNA for dengue virus typing through real-time reverse transcription-polymerase chain reaction

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ARTICLE INFO

*Keywords:*
Serotyping
qRT-PCR
In vitro transcription
Dengue virus

ABSTRACT

*Dengue virus* (DENV) is the causative agent of one of the most important febrile illnesses worldwide. Four DENV serotypes are responsible for a broad clinical spectrum of the disease. Positive controls are costly and required for the validation of molecular test results of DENV serotyping. In this study, we describe the *in silico* design of the qDENV-Control plasmid with the target sequences to oligonucleotides and probes widely used for DENV serotyping, and the subsequent production of qDENV Control RNA by T7-driven *run-off in vitro* transcription. The qDENV Control RNA was successfully used to validate the positive and negative DENV serotyping results, allowing its incorporation in routine in-house protocols for virologic surveillance. This Control RNA allowed the absolute quantification of viral RNA copies from unknown samples as required in several fundamental studies.

## 1. Introduction

Dengue is a febrile disease of major public health concern in the tropical and sub-tropical world with an estimated of 50–100 million infections each year (WHO, 2012), reaching 390 million infections when cartographic approaches are used to estimate mild ambulatory or asymptomatic infections (Bhatt et al., 2013). Severe dengue (SD) is observed in approximately 10% of dengue cases in more than 100 countries (Guo et al., 2017).

*Dengue virus* (DENV) is the etiological agent of this disease and belongs to the family *Flaviviridae* and the genus *Flavivirus*. This virus complex is composed of four closely-related viruses (DENV-1 to -4) with a single-stranded positive polarity RNA genome (ssRNA+) of around 10.7 kb, encoding for a viral polyprotein that is subsequently cleaved to produce the structural [capsid (C), membrane (M) and envelope (E)], and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Lindenbach et al., 2013).

The four antigenically distinct serotypes co-circulate in most of DENV endemic countries. DENV detection and serotyping has become important at the epidemiologic surveillance level for predicting the population immune history and susceptibility, as the most widely accepted hypothesis to explain the progression to severe dengue is the antibody-dependent enhancement (Halstead, 2015). Antibodies generated during a primary infection with a specific serotype may cross-react (but not neutralize) with a different serotype during a secondary infection, enhancing the virus internalization and subsequent replication (Halstead, 2015).

Nucleic acid detection techniques have shown excellent results for the rapid identification of DENV genomic RNA with high sensitivity during the acute phase of the disease. Several reverse transcription-polymerase chain reaction (RT-PCR) protocols for specific detection and serotyping of DENV have been developed since the early 90's (Harris et al., 1998; Lanciotti et al., 1992; Usme-Ciro et al., 2012), increasing our understanding of the disease dynamics (Guo et al., 2017).

Real-time or quantitative RT-PCR assays (qRT-PCR) with extremely high sensitivity and specificity have been available for DENV molecular diagnosis and surveillance from early 2000's (Callahan et al., 2001; Chien et al., 2006; Johnson et al., 2005; Leparc-Goffart et al., 2009). Johnson et al., reported the development of a fourplex DENV serotype-specific real-time nucleic acid detection assay (Johnson et al., 2005). This assay targeted to NS5, E, M and M/E regions of DENV-1 to -4, respectively, has been extensively used for DENV detection and typing and the original design of primers and probes with some degenerations for polymorphic sites has been implemented in the CDC DENV-1-4 Real-

*Abbreviations:* CT, cycle threshold; DENV, dengue virus; RT-PCR, reverse transcription-polymerase chain reaction

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https://doi.org/10.1016/j.jviromet.2019.113677
Received 31 January 2019; Received in revised form 5 June 2019; Accepted 9 June 2019
Available online 10 June 2019

Time RT-PCR Assay (Santiago et al., 2013); Although a positive control for this assay was developed by mixing heat inactivated DENV-1 to -4 standards (https://www.cdc.gov/dengue/clinicallab/realtime.html), its availability to validate the performance of each serotype-specific fluorogenic probe for in-house protocols is limited. This is largely due to lack of availability of expensive biosafety facilities in some routine diagnostics laboratories.

In this study, we evaluated the utility of an *in vitro*-transcribed control RNA containing the target sequences for each of the four DENV serotypes for the validation of real-time RT-PCR results. To assess this, the target sequences for specific primers and probes were cloned in the pBluescript II KS (+) phagemid, which was later used for generation of large amounts of a control RNA by T7-driven *in vitro* transcription. This assay allowed the detection of the four serotypes from the *in vitro*-transcribed DENV control RNA. In addition, it was possible to generate standard curves with high efficiency, which allowed the serotyping and quantification of the viral genome copies for the tested samples.

## 2. Materials and methods

### 2.1. In silico design of the control plasmid

The plasmid was designed as a positive control for T7-driven *in vitro* transcription and subsequent use in the DENV serotyping through real-time RT-PCR. Four target sequences complementary to the serotype-specific probes and primers of the previously described DENV serotyping assay (Johnson et al., 2005; Santiago et al., 2013) were assembled in a single molecule through the SeqBuilder module of the LaserGene® version 7.2.1 (DNASTAR Inc. 2007; Madison, WI, USA.). Restriction sites were rationally chosen and used as spacers at the intersections of each serotype-specific fragment and to facilitate the modification of target sequences, according to the accumulation of genetic variability in the circulating viruses over time. The selected restriction sites (*Sac I, Sma I, Bam HI, Hind III and Kpn I*) were present in the MCS of the cloning vector (*pBluescript II KS* +) and absent in the four serotype-specific regions to be included in the construct. The target oligonucleotide and probe sequences (5'–3') were assembled in the following order: D1-F (caaaaggaagtcgtgcaata); D1-Probe (catgtggttggagcacgc); D1-R (ctgagtgaattctctctactgaacc); D2-F (caggttatggcactgtcacgat); D2-Probe (ctctccgagaacaggcctcgacttcaa); D2-R (ccatctgcagcaacaccatctc); D3-F (ggactggacacacgcactca); D3-Probe (acctggatgtcggctgaaggagcttg); D3-R (catgtctctaccttctcgacttgtct); D4-F (ttgtcctaatgatgctggtcg); D4-Probe (ttcctactcctacgcatcgcattccg) and D4-R (tccacctgagactccttcca) (Fig. 1).

### 2.2. Generation of the insert by gene synthesis

Once the construct was designed, gene synthesis and cloning in the vector *pBluescript II KS+* was performed by the GenScript® company. This vector was chosen as it contained the T7 promoter upstream of the multiple cloning site (MCS), which was necessary for the subsequent *in vitro* transcription to produce control RNA. The new construct, named pBlue-qDENV-Control plasmid was quantified, used for transformation of ultracompetent *E. coli* DH5α, following standard molecular biology procedures (Sambrook and Russell, 2006). The pBlue-qDENV-Control plasmid is available upon request to the corresponding author.

### 2.3. Plasmid propagation and purification through miniprep

Transforming colonies growing in selective LB agar plates with ampicillin were picked, transferred to a volume of 6 ml of LB plus ampicillin and incubated overnight at 37 °C, with shaking at 180 rpm. Subsequently, 800 µl of each bacterial culture were mixed with 200 µl of pure sterile glycerol and glycerol stocks stored at −80 °C. The remaining volume of the culture was used for plasmid purification, using the QIAprep Miniprep commercial kit (Qiagen Inc., Chatsworth, CA, USA).

### 2.4. Run-off in vitro transcription

To obtain the control RNA containing the sequences of the four DENV serotypes that could be used as positive control of the reverse transcription and polymerase chain reaction during the real-time RT-PCR assay, the pBlue-qDENV-Control plasmid and the commercial T7 RiboMAX™ Express Large-Scale RNA Production System (Promega Corp., Madison, WI, USA) were used, following the manufacturer recommendations. 1 µg of the plasmid was linearized with 10 U of the *Sap* I restriction endonuclease, which cut in a region around 391 bp downstream to the DENV-1 to -4 insert sequence and generates a 5' overhang end, which is indispensable to avoid the formation of non-specific transcripts. The linearized plasmid was purified through the QIAQuick PCR Purification kit (Qiagen In., Chatsworth, CA, USA) and 130 ng of the purified product were subsequently mixed with 10 µl of the RiboMAX™ Express T7 2X buffer and 2 µl of the T7 Express Enzyme mix and incubated at 37 °C for 1 h, followed by 65 °C for 20 min and stored at 4 °C. The elimination of the template DNA was carried out by incubating at 37 °C for 15 min with 2U RQ1 RNase-free DNase I for up to 200 ng of plasmid DNA in a final volume of 20 µl. The subsequent qDENV Control RNA was purified using the QIAamp® Viral RNA Mini Kit (Qiagen Inc., Chatsworth, CA, USA), quantified using a NanoDrop 2000 spectrophotometer and stored at −80 °C.

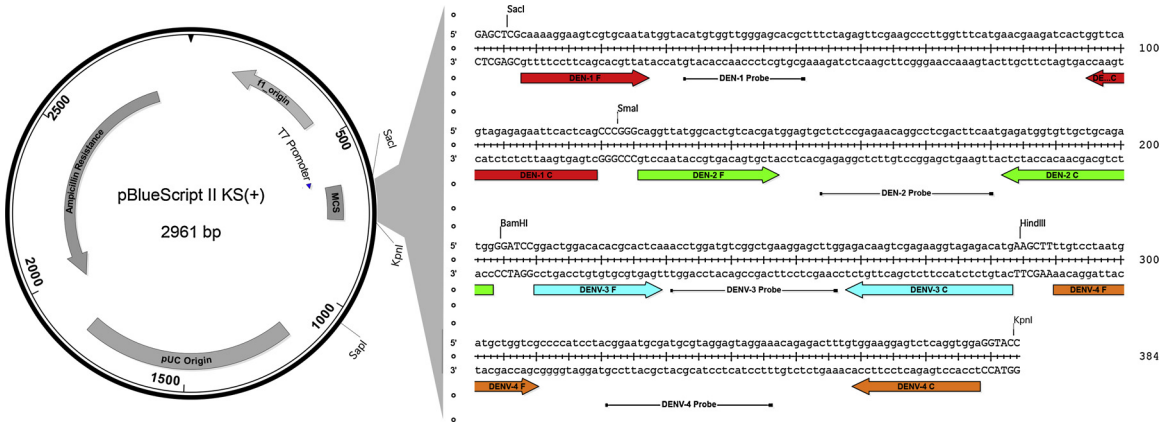


**Fig. 1. *In silico* design of the pBlue-qDENV-Control plasmid**. The sequences of previously reported primers and probes specific for each DENV serotype were assembled in a single molecule with the cleavage sites for the restriction endonucleases *Sma* I, *Bam* HI and *Hind* III used as spacers for future modifications and *Sac* I and *Kpn* I used for cloning in the pBluescript II KS(+) phagemid.

### 2.5. qDENV control RNA extract verification and standard curve generation

To evaluate the complete elimination of the plasmid DNA from the qDENV control RNA extracts and to validate the reverse transcription (RT) step, one-step, and two-step real-time RT-PCR assays using RQ1 DNase I-treated and non-treated RNA extracts were performed with and without the addition of the reverse transcriptase. Lower CT values were expected for the DNase (−) RT (+) assays, mid-range CT values for the DNase (−) RT (−) assays, higher CT values for the DNase (+) RT (+) assays, and finally no amplification was expected for the DNase (+) RT (−) assay. For the two-step RT-PCR the M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA) was used according to the manufacturer's instructions. The RT reaction was prepared by mixing 50 ng of random hexamers, 500 nM of each dNTP and 5 µl of the $1{,}5 \times 10^{-4}$ ng/µl ($3{,}4 \times 10^{5}$ GCE/µl) dilution of the qDENV Control RNA, incubating at 65 °C for 5 min and immediately transferring to a 4 °C cooler. Subsequently, 4 µl of 5X First-Strand buffer, 10 mM of DTT, 10 U of RNaseOUT™ Recombinant Ribonuclease Inhibitor and 200 U of M-MLV RT were added and incubated at 25 °C for 10 min, followed by 37 °C for 50 min and 70 °C for 15 min. The PCR was prepared by following the instructions of the TaqMan® Universal PCR Master Mix (Life Technologies, Carlsbad, CA, USA), and DENV-1 to -4 primer and probes concentrations per reaction were adjusted as previously reported (Johnson et al., 2005); and 5 µl of the cDNA. The assay was run in the Applied Biosystems® 7500 Real-Time PCR instrument. For the one-step RT-PCR, the SuperScript™ III Platinum® Reaction One-Step qRT-PCR Kit (Life Technologies, Carlsbad, CA, USA) was used by following the manufacturer's recommendations and the previously described amounts of primers and probes. To evaluate the efficiency and analytical sensitivity of the RT-PCR assay, a standard curve was generated by molecular amplification from 10-fold serial dilutions of the qDENV control RNA covering the range of $3{,}42 \times 10^{-1}$–$3{,}42 \times 10^{8}$ genome copy equivalents (GCE)/µl, in 8 replicates at each concentration. The limit of detection (LOD) at 95% probability was calculated with Probit regression model, using IBM-SPSS software (version 19).

### 2.6. Performance of the qDENV control RNA for absolute quantification

Supernatants of VERO cells infected with DENV strains corresponding to the four serotypes (previously isolated from human serum samples referred to the Virology Laboratory at the INS, Colombia for virologic surveillance) were collected at one to five days post-infection and used for viral RNA extraction through the QIAamp® Viral RNA Mini Kit (Qiagen Inc., Chatsworth, CA, USA) and plaque assay for quantification of plaque forming units (PFU)/mL in VERO cell monolayers as previously described (Martinez-Gutierrez et al., 2014). Absolute quantification of the number of viral RNA copies was performed by using a standard curve of the qDENV Control RNA as described above.

## 3. Results

### 3.1. Successful generation of qDENV-control RNA by run-off in vitro transcription

After linearization of the *in silico*-designed and chemically-synthetized pBlue-qDENV-Control plasmid with the restriction endonuclease *Sap* I, it was purified and used for T7-driven *in vitro* transcription, allowing the production of the qDENV Control RNA. This control RNA/plasmid DNA mix (1536 ng) was subsequently treated with DNase I and 2 ng/µl-aliquots stored for subsequent assays. The complete plasmid DNA elimination was corroborated by the absence of amplification for the DNase (+) RT (−) experimental point (Table 1). The DNase (+) RT (+) assay allowed to detect specific amplification in each one of the channels of the qRT-PCR system.

A standard curve is necessary to assess the PCR amplification efficiency (measured as a percentage) (Ruijter et al., 2009). An efficiency of 100% is reached when the PCR amplicons double in quantity after each cycle during the geometric phase. However, a PCR efficiency of exactly 100% is rarely observed, since reactions are susceptible to internal and external factors that can influence the amplification including the quality of the reagents, the residual activity of the reverse transcriptase, the precision of the instruments used, and the proper handling/dilution of the sample (Livak and Schmittgen, 2001; Suslov and Steindler, 2005). Serial dilutions of the qDENV Control RNA allowed to estimate efficiency of the qRT-PCR with values of 93.7, 93.1, 91,9 and 96.3% for DENV-1 to -4, respectively, using 8 replicates per dilution (Fig. 2). The linearity of the method was assessed by the linear behavior through a broad dynamic range (6 logs). The LOD was determined to be between $4.1 \times 10^{2}$ and $2.9 \times 10^{4}$ GCE/ml (Fig. 3). These results demonstrate the successful generation of the qDENV Control RNA that can be used to validate the results obtained for every target serotype through a four-channel qRT-PCR system and is therefore useful for DENV molecular detection and serotypes surveillance. RNA aliquots should be properly manipulated to avoid degradation by following the standard recommendation for RNA handling, including the use RNase-free materials/reagents and the storage at ultra-low temperature avoiding repetitive freezing/thawing.

**Table 1**
CT values obtained in the real-time RT-PCR assay using the qRT-PCR-DENV Control RNA.

| ASSAY | CT value (Mean) | | | |
|---|---|---|---|---|
| | DENV-1 | DENV-2 | DENV-3 | DENV-4 |
| DNase (−) RT (+) | 13.81 | 10.69 | 11.40 | 8.62 |
| DNase (−) RT (−) | 15.62 | 14.30 | 15.66 | 16.19 |
| DNase (+) RT (+) | 24.52 | 26.18 | 25.26 | 26.03 |
| DNase (+) RT (−) | Undet. | Undet. | Undet. | Undet. |
| NTC | Undet. | Undet. | Undet. | Undet. |

RT: Reverse Transcriptase. NTC: No Template Control. Undet: Undetermined.

### 3.2. The qDENV control RNA allowed the absolute quantification of DENV RNA copies in unknown samples

Serotype-specific standard curves generated as described above, allowed the interpolation of the CT values of unknown samples for their absolute quantification. Amplification was observed for all the samples derived from culture supernatants of time-matched points of DENV-1 to -4 growth curve assays. DENV-2 and -4 showed higher number of genome copies from day 1 to day 3 reaching the order of ˜10 logs at day 4, while DENV-3 showed a very low number of genome copies at 1 dpi that progressively increased to reach the maximum at 5 dpi. Finally, DENV-3 displayed a little change (3 logs difference) in the number of genome copies during the time course, reaching the higher number at 5 dpi (Fig. 3). When the viral titers of PFU/ml were compared to GCE/ml, the later showed 3 to 5 log10 higher than the number of infectious particles determined by PFU assays which was in the expected range (Fig. 4) (van der Schaar et al., 2007). Together, these results demonstrate the utility of this novel molecular control for serotyping and for absolute quantification of DENV RNA copies from unknown samples.

## 4. Discussion

DENV molecular detection and serotype surveillance is mandatory to reconstruct the DENV dynamics at the epidemiologic level (Katzelnick et al., 2018) and has been implemented in the virologic surveillance protocols at country level and requested by the Pan American Health Organization (PAHO/WHO) for regional surveillance (http://www.paho.org/data/index.php/en/mnu-topics/indicadores-dengue-en.html). The circulation of a specific DENV serotype during an epidemic is important to assess the potential clinical impact of future

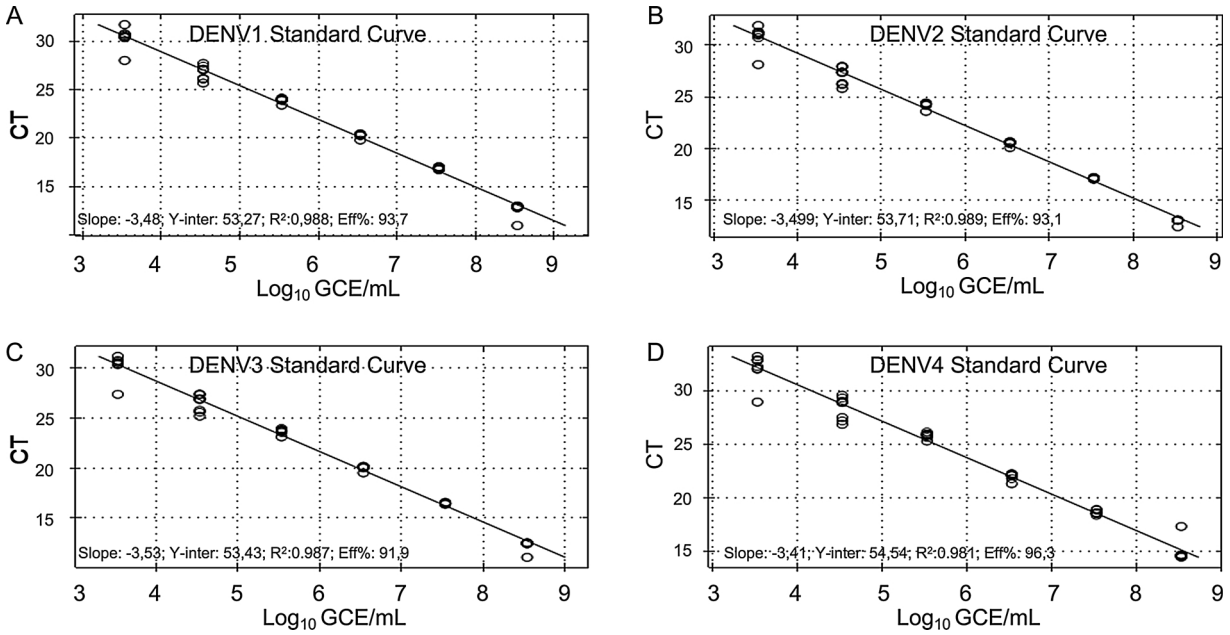


**Fig. 2. Serotype-specific DENV qRT-PCR performance using the qDENV Control RNA**. Standard curves showing efficiency and linearity obtained from six serial dilutions of the qDENV Control RNA through specific channels for each target/serotype. A–D) DENV-1 to -4, respectively. Every experimental point in the dilution series was supported by eight replicates.

epidemics, as in some settings it can predispose the individuals to more severe clinical outcomes.

Virus isolation combined with indirect immunofluorescence using serotype-specific antibodies was the gold standard for DENV serotype assessment since early 80's (Henchal et al., 1982). More recently, qPCR has become the most popular technique in molecular diagnostics because it is highly sensitive, and it allows the absolute or relative quantification of the amount of starting material (*e.g.* genome copies) (Livak and Schmittgen, 2001). RT-PCR tests for DENV detection and serotyping emerged and increased during the last two decades and led to the need for positive controls to validate the results and efficacy of the assays.

An inactivated DENV mix containing the four serotypes is commonly prepared from reference strains propagated in cell cultures, requiring specific facilities and personnel qualified in virologic techniques that are not frequently used in diagnostic laboratories from middle- to low-income countries, limiting its scalability and widespread use. The strategy presented here differs from the direct use of plasmid DNA as positive controls (Maaroufi et al., 2006), because the qDENV Control RNA was *in vitro* synthetized, allowing the validation of the critical RT

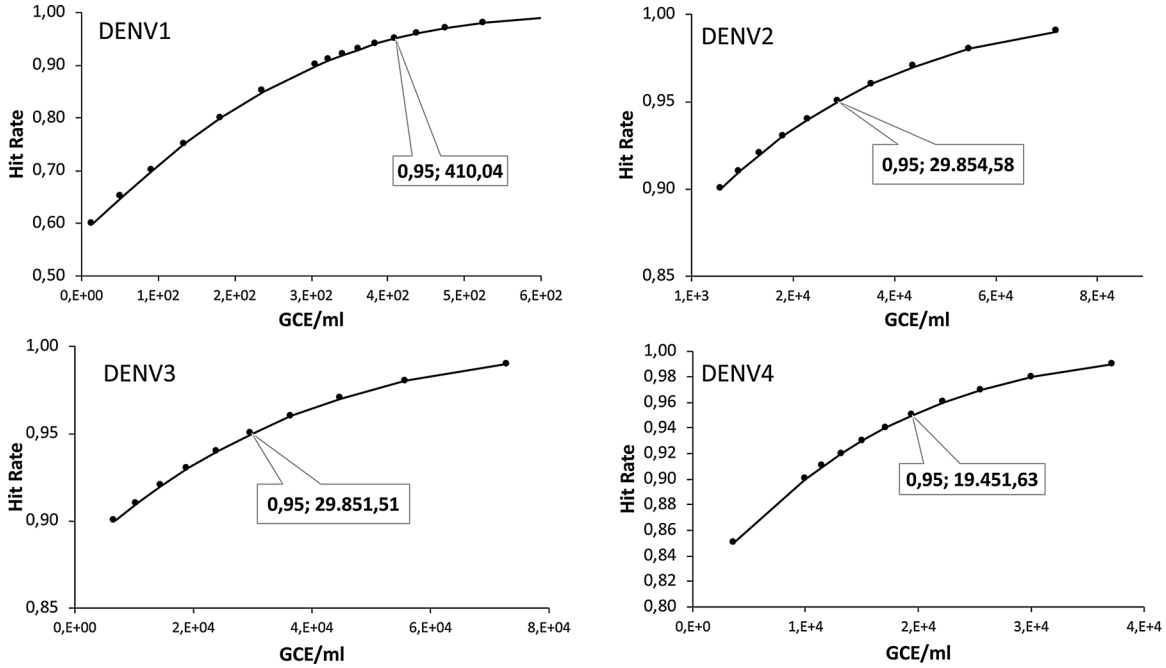


**Fig. 3. Probit regression analysis of DENV1-4 GCE/µL through real time RT-PCR**. Ten-fold serial dilutions of the qDENV control RNA in 8 replicates per dilution were used for the analysis. The hit rate (or percent detection) is plotted against the GCE/mL. The 95% hit rate and the corresponding LOD is indicated in the dialog box for each serotype.

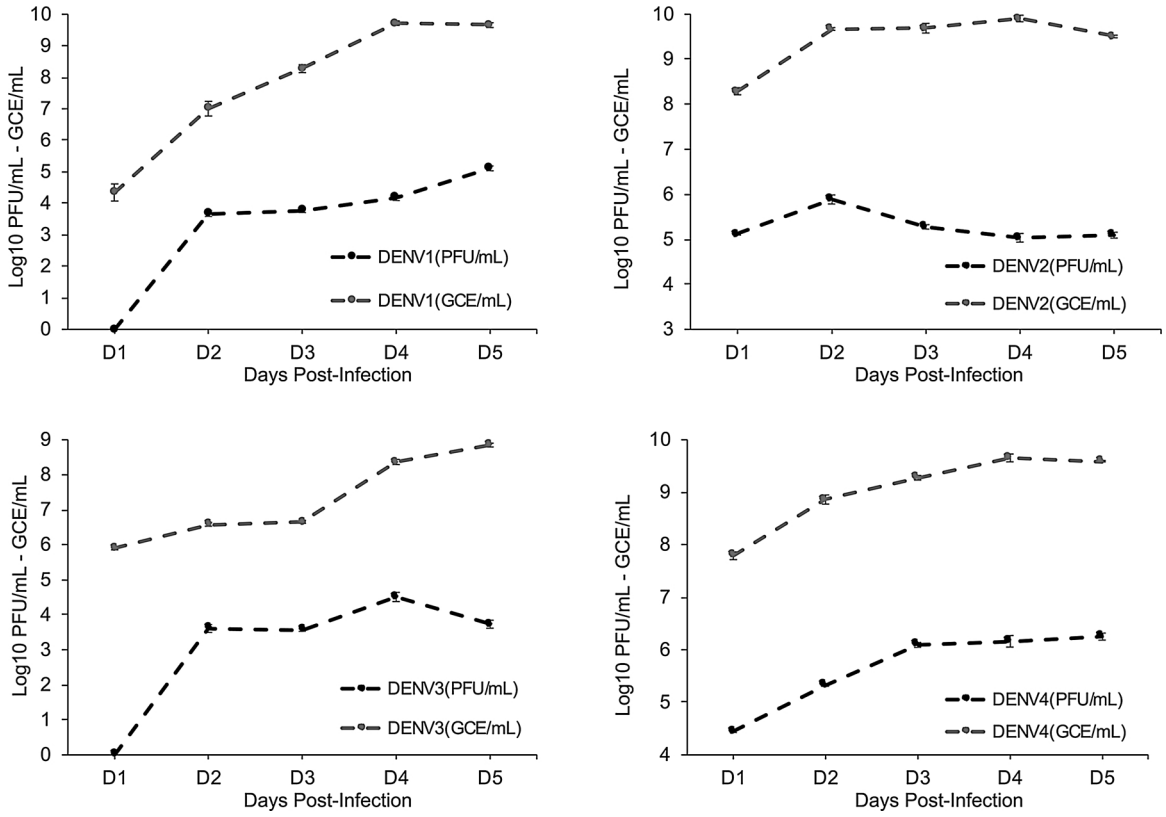


**Fig. 4. Viral growth curves of DENV strains from serotypes 1 to 4**. PFU/ml and GCE/ml were estimated in cell culture supernatants at 1 to 5 days post-infection by plaque assay and qRT-PCR, respectively. Values correspond to the mean PFU/ml and GCE/ml, after $Log_{10}$ transformation. Error bars indicate standard deviation of 3 replicates.

step of the qRT-PCR protocol. Having confirmed the complete elimination of plasmid DNA after DNase I treatment, this approach demonstrated to be successful to validate the detection and typing of DENV through qRT-PCR. The strategy can be applied in the absence of virologic expertise and replicated to the development of molecular tests for the detection of other RNA viruses of medical importance for which the availability of positive controls is limited.

One important use of the here developed control is the direct serotyping from clinical samples as originally suggested for the qRT-PCR assay (Johnson et al., 2005; Santiago et al., 2013). Viremia levels during the acute phase of DENV infection are variable, depending on the immune status, days from onset of symptoms and the presence of coinfections; commonly are in a range from $10^3$ to $10^6$ PFU/ml (around 3 logs higher if it is expressed in terms of GCE). The LOD and wide detection range obtained here comprises the viremia titers commonly found in clinical samples.

The *in vitro*-transcribed qDENV Control RNA can also be used for the absolute quantification of viral RNA copies of each DENV serotype in unknown samples through a standard curve with serial dilutions of known quantities of the control RNA (Pfaffl, 2004). Quantitative results are commonly obtained in research laboratories for reconstruction of viral growth curves as part of fundamental studies asking for the viral contribution to the virulence and pathogenesis (Usme-Ciro et al., 2014).

The publication of the sequences of primers and probes incorporated to the CDC DENV-1-4 Real-Time RT-PCR Assay (Santiago et al., 2013) allowed to corroborate almost complete correspondence with those previously described (Johnson et al., 2005), and to theoretically predict the same performance of the qDENV Control RNA with the new set of primers and probes. Therefore, the qDENV Control RNA has the potential to be incorporated to the previously described assay mainly where in-house protocols are required.

## 5. Conclusions

A plasmid construct containing the primers and probes sequences of the four DENV serotypes was *in silico* designed and used to generate the qDENV Control RNA by T7-driven *in vitro* transcription. This control allowed to avoid the expensive manipulation of virus strains in cell culture laboratories with restrictive biosecurity requirements and the costly calibration of each serotype at equimolecular quantities for a good performance during the test. This study is expected to contribute to the improvement of the DENV detection and serotype surveillance through qRT-PCR, allowing to validate the detection and typing of each serotype independently using a unique qDENV Control RNA. It also facilitates the establishment of in-house protocols that are the preferred option in several research laboratories where virus quantification could be the most important application of the present development.

## Conflict of interest

The authors declare no conflict of interest.

## Funding

This work was supported by COLCIENCIAS (grant 210465740977 to J.A. Usme-Ciro) and the Colombian National Institute of Health.

## Acknowledgments

To the Virology Lab at the Instituto Nacional de Salud, Colombia, for providing the previously diagnosed and serotyped samples, and the Sequencing and Genomics Unit team for helpful discussions.

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