Development of Synthetic Molecular Standards for Dengue Virus

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Background & Introduction

Dengue fever is an acute illness caused by any one of four serotypes (1-4) of genetically related dengue viruses (DENV), with an estimated 390 million cases reported annually. Currently, quantitative RT-PCR (qRT-PCR) is the preferred method for the detection and quantification of DENV in clinical diagnostics and epidemiological surveillance. The accuracy of a qRT-PCR assay relies on the generation of a standard curve using a positive control with a known viral genome concentration.

Native DENV RNA can be used as a standard for these assays; however, the full-length dengue viral RNA is on the Commerce Control List and requires a permit from the US Department of Commerce for international shipment. To make DENV RNA standards more accessible, ATCC has developed four synthetic molecular standards that represent DENV serotypes 1-4. Each standard contains short fragments from the capsid, membrane, and envelope genes of the DENV genome, as well as target regions encompassing the primer sequences from numerous published RT-PCR assays, including the DENV-1-4 Real-Time RT-PCR Assay developed by the CDC[1]. The synthetic RNA standards were quantified by Droplet Digital™ PCR (ddPCR™) in order to package precise copies of RNA. Moreover, given the inherent labile nature of RNA, a stabilization matrix was added to the quantitated RNA preparation. As compared to native RNA, these synthetic standards are easier to use as controls for qRT-PCR assays, exhibit less variability, have a longer shelf life, eliminate the need to culture viruses and can be used under BSL-1 conditions. Further, this synthetic quantitative RNA approach can be extended to other pathogenic viruses which are unculturable or need to be grown in a high-containment facility.

In the following proof-of-concept study, we amplified the synthetic molecular standards with the published primers from the CDC assay[1] and Waggoner *et al*[2] , and used the generated standard curves to quantify viral RNA extracted from various DENV strains.

Materials and Methods

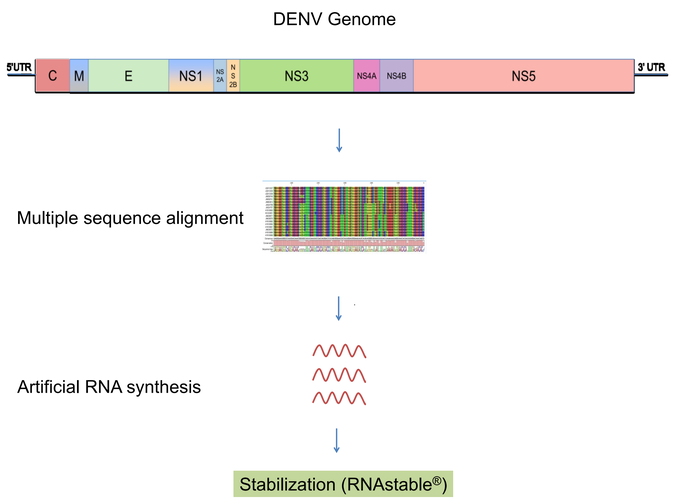
Viruses:
The following DENV strains, representing DENV serotypes 1, 2 and 4 were quantified using the standard curves generated from the DENV 1, 2 and 4 synthetic molecular standards, respectively:

Dengue virus 1 = TH-S-man (ATCC® VR-1586™)
Dengue virus 2 = New Guinea C (NR-84, BEI Resources)
Dengue virus 4 = H241 (ATCC® VR-1257™)

Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN®). Viral RNA was diluted 1:100, 1:1000, and 1:10,000 for the qRT-PCR assay. Uninfected Vero (ATCC® CCL-81™) and LLC-MK2 Derivative (ATCC® CCL-7.1™) cell lines were used as negative controls in the qRT-PCR assays.

qRT-PCR assay:
qRT-PCR assays were performed according to instructions provided[1], with slight modifications, using the CFX96™ Real-Time PCR Detection System (Bio-Rad®). Two different primer sets were employed to examine each individual Dengue serotype using the same conditions for both: the commercial primer set included in the CDC Real-Time RT-PCR assay[1] and the primer set published by Waggoner *et al.*[2]. Cycling conditions for all primer sets were 50ºC for 15 min and 95ºC for 2 min, followed by 45 cycles of 95ºC for 15 sec and 60ºC for 30 sec. The exception was for the DENV-4 published primer set; the annealing temperature was 55ºC for 30 sec. Standard curves were generated using serial ten-fold dilutions of the synthetic RNA standards, ranging from 1 copy to 1 x 10^6 copies/µL. RNA samples and standards were tested in triplicate. The relative fluorescence unit (RFU) baseline threshold was calculated using CFX Manager™ 3.0 Software (Bio-Rad).

ATCC Design Strategy

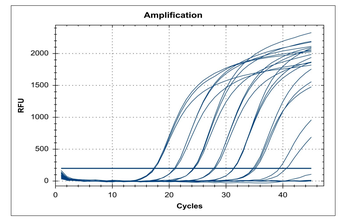


ATCC Synthetic Molecular Standards	
DENV-1	ATCC® VR-3228SD™
DENV-2	ATCC® VR-3229SD™
DENV-3	ATCC® VR-3230SD™
DENV-4	ATCC® VR-3231SD™

Advantages	Applications
• ATCC fully authenticated & characterized Genuine Nucleics • Consistent and accurate results • BSL-1 ready-to-use control • No shipping restrictions • Quantitative format • Stabilized RNA	• Generation of a standard curve for quantitative RT-PCR (qRT-PCR) • Positive control for RT-PCR assays • Independent standard for validation and verification studies • Monitor assay-to-assay and lot-to-lot variation

Generation of Standard Curves from DENV Synthetic Molecular Standards

A



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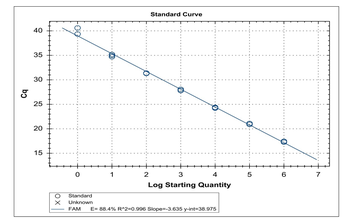


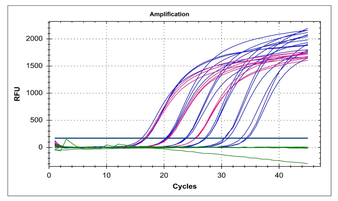
C

Primer and Probe		DENV-1	DENV-2	DENV-4
CDC Assay[1]	Slope	-3.244	-3.277	-3.642
	R^2	0.99	0.996	0.996
Published Assay[2]	Slope	-3.536	-3.535	-3.775
	R^2	0.991	0.997	0.996

Figure 1. An example of **A)** amplification plot and **B)** standard curve generated using the DENV-4 molecular standard (ATCC® VR-3231SD™) in conjunction with the CDC primer and probe set[1]. **C)** The slope and R^2 values generated using DENV 1, 2 and 4 molecular standards with the primer and probe sets from the CDC DENV-1-4 Real-Time RT-PCR Assay[1] and Waggoner *et al.* assay[2].

Quantification of Native DENV-1, 2 and 4 Using Synthetic Molecular Standards

A



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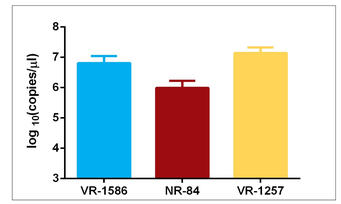

Figure 2. A) An example of qRT-PCR amplification plot showing DENV-4 synthetic molecular standards (blue) and the native sample of unknown concentration (pink). Unknown samples were serial diluted ten-fold and run in triplicate wells. **B)** Copy numbers of DENV-1, 2 and 4 samples as determined by the qRT-PCR standard curves generated by using DENV (1, 2 and 4) molecular standards respectively.

References

1. CDC. DENV-1-4 Real-Time RT-PCR Assay for Detection and Serotype Identification of Dengue virus. Instructions for Use Package Insert, http://www.cdc.gov/dengue/resources/rt_pcr/CDCPackageInsert.pdf.
2. Waggoner JJ, *et al*. Single-reaction, multiplex, real-time RT-PCR for the detection, quantitation, and serotyping of dengue viruses. PLoS Negl Trop Dis 7(4): e2116, 2013.

Disclaimers