Development of Synthetic Molecular Standards for Dengue Virus

Shamaila Ashraf, Melisa Wilson, Afshin Sohrabi, Stephen King, Brian Chase, Dev Mittar, Kurt Langenbach and Andrew G. Cawthon

Background & Introduction

Dengue fever is an acute illness caused by any one of four serotypes (1-4) of generally 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 to 4 Real-Time RT-PCR Assay developed by the CDC. 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* and Waggoner et al.**, 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:

- Dengu virus 1 = TH-S-man (ATCC® VR-1567™)
- Dengu virus 2 = New Guinea C (NR-84, BEI Resources)
- Dengu virus 4 = H241 (ATCC® VR-1297™)

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-Md2 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†, with slight modifications, using the CFX360™ 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 and the primer set published by Waggoner et al.‡. Cycling conditions for all primer sets were 50°C for 15 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec. The exception was for the DENV-4 published primer set; the annealing temperature was 55°C for 30 sec. Standard curves were generated using serial tenfold dilutions of the synthetic RNA standards, ranging from 1 copy to 1 x 10^10 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).

Advantages:
- ATCC fully authenticated & characterized Genuine Nucleics
- Consistent and accurate results
- BSL-1 ready-to-use control
- No shipping restrictions
- Quantitative format
- Stabilized RNA

Applications:
- 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

References:

Disclaimers:
Bio-Rad®, CFX360™, Droplet Digital™/PCR, Real-Time PCR Detection System, and CFX Manager™ 3.0 Software are registered trademarks or trademarks of Bio-Rad Laboratories, Inc. RNAstand™ is registered trademark of Bimotrica, Inc. QIAamp® Viral RNA Mini Kit and QIAEN® are registered trademarks of the QIAGEN Inc. The following reagents were obtained through BEI Resources, NIAID, NIH. Dengue Virus Type 2, New Guinea C, NR-84.

Generation of Standard Curves from DENV Synthetic Molecular Standards

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; C) The slope and R² 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* and Waggoner et al.**.

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

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 1:4-fold and run in triplicate wells. B) Copy numbers of DENV-1, 2 and 4 as determined by the qRT-PCR standard curves generated by using DENV 1, 2 and 4 molecular standards respectively.