Development and Validation of Synthetic Molecular Standards for Hepatitis A, B, C, and E Viruses

Britany Tang, BS, Michael Geimer, BS, Kyle Young, MBA, and Dev Mittar, PhD | ATCC, Manassas, VA 20110

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Background and Introduction

The Hepatitis A (HAV), B (HBV), C (HCV), and E (HEV) viruses are the most common causes of acute and chronic liver disease, which is a major source of morbidity and mortality worldwide. Currently, real-time PCR assays are routinely used to ensure the rapid detection and quantification of these viruses; however, the accuracy and reproducibility of these assays are limited by the lack of precisely quantified control materials, which are essential for determining analytical specificity and sensitivity. To address this need, ATCC has developed synthetic quantitative molecular standards for HAV, HBV, HCV, and HEV that are compliant with ISO13485, quantified via digital PCR, and validated with published qRT-PCR assays.[1,2,3,4] In the following proof-of-concept study, the HAV, HBV, HCV, and HEV synthetic molecular standards were used to generate standard curves with published primer sets.[1,2,3,4] Furthermore, the standard curves for HBV and HCV were used to quantify the 3rd WHO international standard for HBV and the 4th WHO international standard for HCV from the National Institute for Biological Standards and Control (NIBSC) via the same specific published assays.[1,2]

Material and Methods

Reagents:

The 3rd WHO international standard for HBV (NIBSC code 10/264) contained 8.5 x 10^5 IU/mL and the 4th WHO international standard for HCV (NIBSC code 06/102) contained 2.6 x 10^5 IU/mL. The WHO standards were reconstituted in 0.5 mL of molecular grade water according to the manufacturer's recommendations. Viral DNA/RNA was extracted via QIAamp® Viral RNA Mini Kit (QIAGEN®).

Quantitative Synthetic DNA/ RNA:

Using a proprietary method, we designed synthetic DNA constructs for HAV (ATCC® VR-3257SD™) and HBV (ATCC® VR-3232SD™) and synthetic RNA constructs for HCV (ATCC® VR-3233SD™) and HEV (ATCC® VR-3258SD™). These standards comprise fragments from various conserved regions of viral genomes, including the 5' UTR, viral capsid proteins (VP1–4), self-cleaving peptide 2A, proteinase 3C, and 3D RNA polymerase for HAV; the precore, core, polymerase, surface, and X regions for HBV; the 5´and 3´UTR for HCV; and the 5' UTR, methyl transferase, Y domain, X domain, helicase, RNA-directed RNA polymerase, and open reading frames 2 and 3 (ORF2 and ORF3) for HEV. Following their construction, the standards were authenticated via next-generation sequencing and quantified via digital PCR (Bio-Rad).

qPCR/ qRT-PCR assay:

qRT-PCR assays were performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad) according to manufacturers' instructions with slight modifications. Standard curves were generated in triplicate for each construct; the synthetic constructs were diluted ten-fold to obtain ranges of 3 copies/reaction to 3x10^5 copies/reaction for HBV and 10 copies/reaction to 1x10^5 copies/reaction for HAV, HCV, and HEV. The resulting standard curves for Hepatitis B and C were used to quantify the viral load of the corresponding WHO international standards from the National Institute for Biological Standards and Control, UK (NIBSC).

Digital PCR Assay:

Digital PCR assays were performed according to the manufacturer's instructions on the QX200™ droplet reader (Bio-Rad) and QuantaSoft™ software 1.7.4.0917 (Bio-Rad) for droplet generation and data analysis. The synthetic constructs were diluted 10-fold to obtain three dilutions of 10^4 to 10^6 genome copy number/µL per dilution, which were quantified in triplicate by digital PCR via the same primers and probes from the published qPCR assays and qRT-PCR assay.[1,2]

References

1. Sun S, et al. Development of a new duplex real-time polymerase chain reaction assay for hepatitis B viral DNA detection. Virol. J. 8: 227, 2011.
2. Lee SC, et al. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J. Clin. Microbiol. 38(11): 4171-4179, 2000.
3. Cormier, Jiemin, and Marlene Janes. "Concentration and detection of hepatitis A virus and its indicator from artificial seawater using zeolite." *Journal of virological methods* 235 (2016): 1-8.
4. Matsubayashi, Keiji, et al. "A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route." *Transfusion* 48.7 (2008): 1368-1375.

ATCC Synthetic Molecular Standards

ISO13485	BSL-1	Quantitative	Stabilized

ATCC Part Number	Product Description
ATCC® VR-3257SD™	Quantitative Synthetic Hepatitis A virus DNA
ATCC® VR-3232SD™	Quantitative Synthetic Hepatitis B virus DNA
ATCC® VR-3233SD™	Quantitative Synthetic Hepatitis C virus RNA
ATCC® VR-3258SD™	Quantitative Synthetic Hepatitis E virus RNA

Applications

- Generation of a standard curve for quantitative RT-PCR
- Positive control for RT-PCR assays
- Assay verification and validation studies
- Monitor assay-to-assay and lot-to-lot variation
- Molecular diagnostics assay development

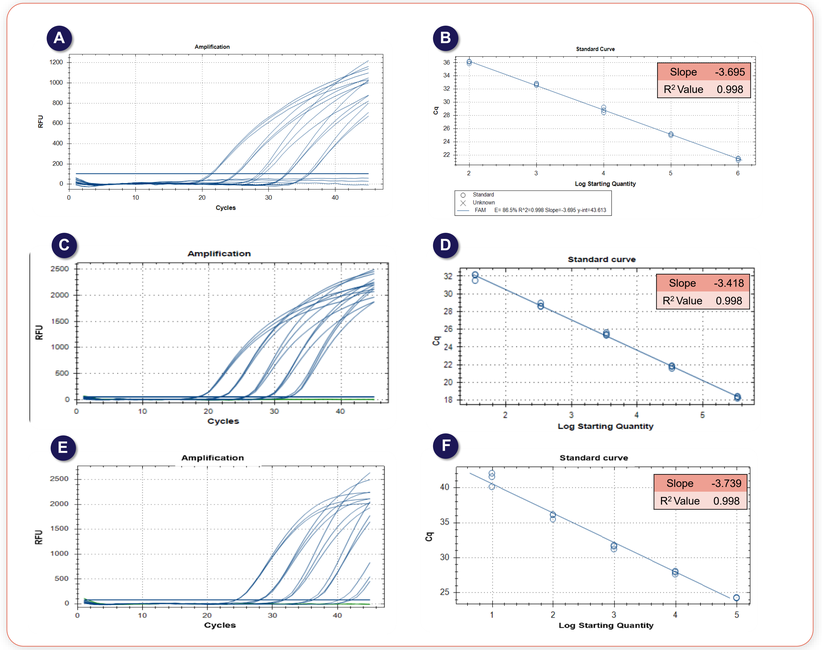

Figure 1. qRT-PCR assay to verify the functionality of the synthetic molecular standard. (A, C, E) Amplification plots and (B, D, F) standard curves were generated with the HAV, HBV, and HCV molecular standards, respectively. The qPCR and qRT-PCR assays were performed as previously described.[1,2,3] Cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 30 sec for HAV; 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 for HBV; and 50°C for 15 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 62°C for 30 sec for HCV. Standard curves were generated by using serial ten-fold dilutions that ranged from 3 copies/reaction to 3x10^5 copies/reaction for HBV and 10 copies/reaction to 1x10^5 copies/reaction for HAV and HCV. The DNA/ RNA standards were tested in triplicate.

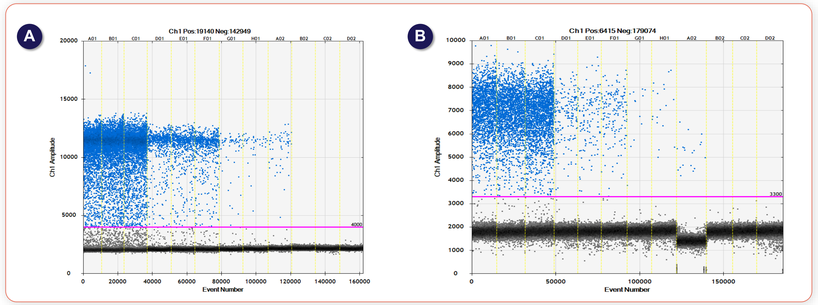

Figure 2. Absolute quantification of HBV and HCV via digital PCR. One-dimensional (1D) amplitude scatter plots of positive and negative digital PCR droplet reactions for 3 dilutions (10^4 to 10^6) in triplicate for (A) HBV and (B) HCV. HBV and HCV were quantified by digital PCR by using the same primers and probe from the published qPCR assay and qRT-PCR assay.[1,2] Digital PCR for HBV was performed as follows: initial denaturation at 95°C for 10 min, amplified 40X at 94°C for 30 sec and 60°C for 1 min, and enzyme deactivation at 98°C for 10 min. ddPCR™ for HCV was performed as follows: reverse transcription at 60°C for 30 min, enzyme activation at 95°C for 5 min, amplified 40X at 94°C for 30 sec and 60°C for 1 min, and enzyme deactivation at 98°C for 10 min. Droplets were analyzed in the QX200 droplet reader. Data were analyzed with QuantaSoft software.

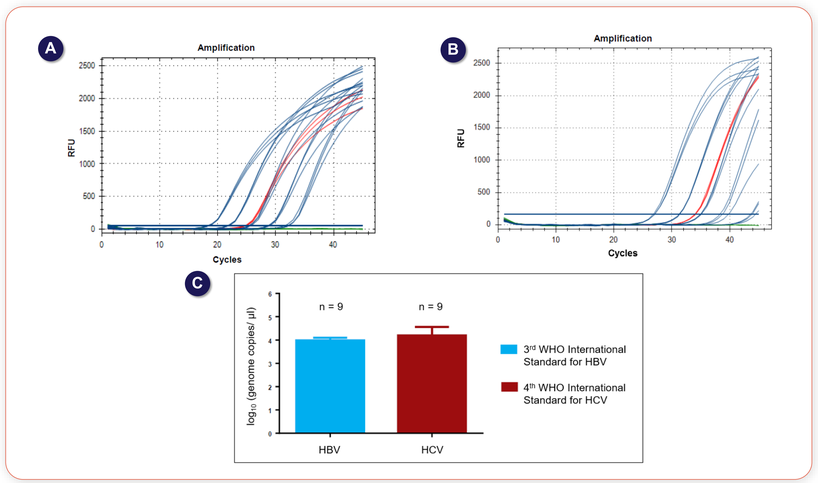

Figure 3. Quantification of WHO international standards for HBV and HCV by using ATCC Synthetic Molecular Standards. An example of a qPCR and qRT-PCR amplification plot generated from the ATCC synthetic molecular standards. (A) HBV (Blue) and the 3rd WHO international standard for HBV (Red) and negative control (green). (B) HCV (Blue) and the 4th WHO international standard for HCV (Red) and negative control (Green). By using the standard curves generated from the ATCC synthetic molecular standards for HBV and HCV under the published assay conditions,[1,2] a value of 9.7 x 10^6 genome copies/mL was assigned to the 3rd WHO international standard for HBV and 1.6 x 10^7 genome copies/mL was assigned to the 4th WHO international standard for HCV. (C) Average genome copies/µL calculated from triplicate wells from triplicate run-to-run qPCR and qRT-PCR experiments for HBV and HCV. Error bars indicate standard deviation and were calculated by using GraphPad Prism software. Both WHO international standards were diluted ten-fold and run in triplicate wells in three independent experiments were used to calculate the quantities of HBV and HCV (genome copies/µL), respectively.

Conclusions

Our proof-of-concept data demonstrates that ATCC quantitative synthetic molecular standards for HAV, HBV, HCV, and HEV can be used as controls for assay development, verification, and validation. These standards can be used to determine the viral load of unknown hepatitis samples through the generation of a standard curve. The standards are compatible with numerous published assays and exhibited minimal variability as evident from the slope and R^2 values. Taken together, these standards provide well-characterized controls for viral detection and quantification.

ATCC 10801 University Boulevard, Manassas, Virginia 20110-2209 **Phone:** 800.638.6597 **Email:** SalesRep@atcc.org **Web:** www.atcc.org