Development of Synthetic Molecular Standards for Hepatitis B and Hepatitis C virus

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Background & Introduction

- Viral hepatitis caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) is a major health concern and affects millions of people worldwide.
- Patients are routinely monitored by quantitative RT-PCR (qRT-PCR) for the presence of HCV RNA or by qPCR for HBV DNA in blood.
- Since these viruses are difficult to culture *in vitro*, obtaining control material for these molecular-based assays is a challenge. To address this problem, ATCC has developed HBV and HCV specific quantitative synthetic molecular standards for use as controls for the detection and quantification of these viruses from clinical samples.
- These synthetic molecular standards include fragments from the polymerase, surface, and X regions for HBV and the 5´and 3´UTR from HCV genome. These synthetic RNA standards are synthesized under ISO 13485:2003 guidance, quantified by Droplet Digital™ PCR (ddPCR™), and stabilized by the addition of RNAstable® and DNAstable®.
- In this study, we used the synthetic molecular standards for HBV and HCV to generate standard curves using published primer sets for HBV[1] and HCV[2].
- Furthermore, we used the respective standard curves 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) using the specific primer sets from published literature[1,2].

Materials and Methods

Reagents:
The following reagents were used in the qRT-PCR assays:

- The synthetic molecular standards for HBV and HCV (ATCC® VR-3232SD™ and ATCC® VR-3233SD™, respectively) were from ATCC.
- The 3rd WHO international standards for HBV (NIBSC code 10/264) and the 4th WHO international standards for HCV (NIBSC code 06/102) were obtained from NIBSC, UK. The 3rd WHO international standards for HBV contained 8.5 x 10^5 IU/ml and the 4th WHO international standards for HCV contained 2.6 x 10^5 IU/ml as assigned by the WHO.
- The WHO standards were reconstituted in 0.5 mL of molecular grade water according to the manufacturer's recommendations.
- Viral DNA or RNA from the respective WHO international standard was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN®). Each dilution was run in triplicate wells and repeated three times in independent experiments run on different days (n=9).

qRT-PCR assay:
qRT-PCR assays were performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad). The primer and probe sets from published qPCR or qRT-PCR assay were used under the following conditions. Cycling conditions for HBV primer sets were 50ºC for 2 min, 95ºC for 2 min, followed by 45 cycles of 95ºC for 15 sec and 60ºC for 30 sec. For HCV, primer set conditions were 50ºC for 15 min and 95ºC for 2 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 30 sec. Standard curves were generated using serial ten-fold dilutions of the respective synthetic molecular standard DNA or RNA, ranging from 3 copies to 3 x 10^5 copies/reaction for HBV and from 10 copies to 1 x 10^5 copies/reaction for HCV. DNA or RNA samples and standards were tested in triplicate. The relative fluorescence unit (RFU) baseline threshold was set automatically and genome copy numbers were calculated using CFX Manager™ 3.0 Software (Bio-Rad).

ATCC® Synthetic Molecular Standards	
ATCC® No.	Description
VR-3232SD	Synthetic Hepatitis B DNA (HBV)
VR-3233SD	Synthetic Hepatitis C RNA (HCV)

Results

Synthetic Molecular standard for HBV (ATCC® VR-3232SD)

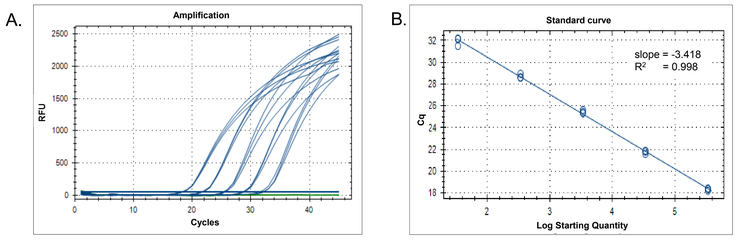

Figure 1. Generation of Standard Curves from Synthetic Molecular Standards for HBV. A.) Amplification plot and **B)** standard curve generated using the HBV synthetic molecular standard (ATCC® VR-3232SD) with the respective primer and probe set from the published Real-Time PCR Assay[1]. (Blue) = Serial ten-fold dilutions of the synthetic molecular standard. (Green) = Negative control.

Synthetic Molecular standard for HCV RNA (ATCC® VR-3233SD)

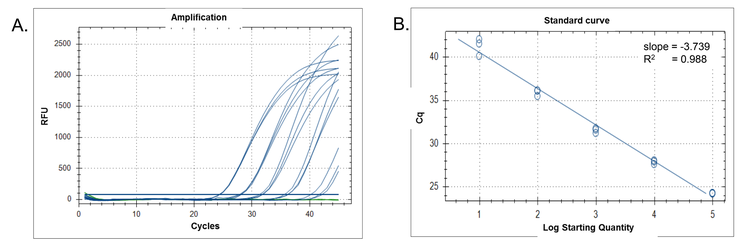

Figure 2. Generation of Standard Curves from Synthetic Molecular Standard for HCV. A.) Amplification plot and **B)** standard curve generated using the HCV synthetic molecular standard (ATCC® VR-3233SD) with the respective primer and probe sets from the published Real-Time RT-PCR Assay[2]. (Blue) = Serial ten-fold dilutions of the synthetic molecular standard. (Green) = Negative control.

Salient features	Applications
• Fully authenticated & characterized ATCC® Genuine Nucleics • Generated under ISO 13485:2003 • Quantitative format • Functional with numerous published assays • BSL-1 ready-to-use control • Stabilized RNA and DNA • Assigned values traceable to WHO international standards	• Generation of a standard curve for qRT-PCR • Positive control for PCR and RT-PCR assays • Independent standard for validation and verification studies • Monitor assay-to-assay and lot-to-lot variation

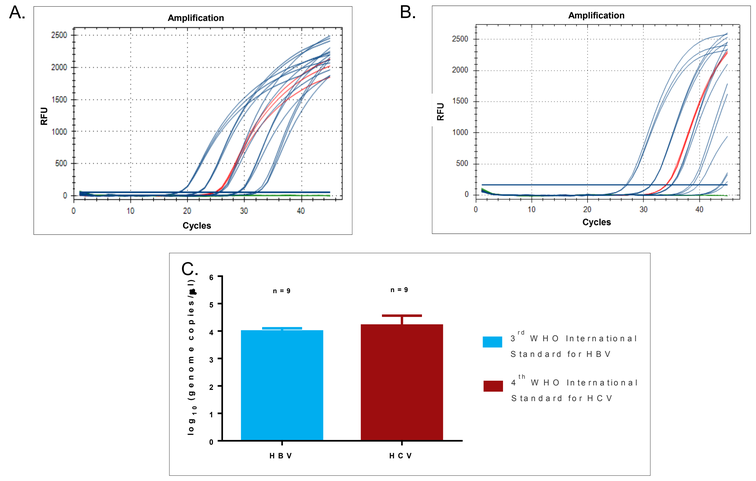

Figure 3. Quantification of WHO international standards for HBV and HCV Using ATCC Synthetic Molecular Standards. An example of a qRT-PCR amplification plot showing the ATCC synthetic molecular standards (Blue) **A)** HBV and the 3rd WHO international standard for HBV (Red). **B)** HCV (Blue) and the 4th WHO international standard for HBV and negative control (Green). Both WHO international standards were diluted ten-fold and run in triplicate wells in three independent experiments (n=9). **C)** Quantitation of the WHO international standards for HBV and HCV samples respectively using the published assays for HBV[1] and HCV[2]. The average from triplicate wells from three independent experiments were used to calculate the quantities of HBV and HCV genomes respectively. Error bars indicate standard deviation and were calculated using GraphPad Prism software.

Conclusions

- The ATCC synthetic molecular constructs provide well-characterized HBV DNA and HCV RNA controls, which are otherwise difficult to obtain due to the unculturable nature of these viruses.
- Using the standard curve generated from the ATCC synthetic molecular standard for HBV under the published assay conditions[1], a value of 9.7 x 10^6 genome copies /mL was assigned to the 3rd WHO international standard.
- Using the standard curve generated from the ATCC synthetic molecular standard for HCV under the published assay conditions[2], a value of 1.6 x 10^7 genome copies /mL was assigned to the 4th WHO international standard.
- By assigning a genome copy per mL value to WHO international standards, a conversion ratio of copies/mL to IU/mL can be easily calculated[3]. In this study, using the specified qPCR and qRT-PCR conditions, we calculated that 1IU/mL is equal to 11.4 and 61.5 genome copies /mL respectively of HBV and HCV synthetic molecular standards.

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. Welzel, et al. Real-time PCR assay for detection and quantification of Hepatitis B virus Genotype A to G. J. Clin. Microbiol. 44(9):3325-3333. 2006.

Disclaimers

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