Development And Validation Of Quantitative Synthetic Molecular Standards For Human Herpesvirus 6A, 7, and 8

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Background and Introduction

Herpesviridae is a large family of double-stranded DNA viruses that have the ability to infect a wide range of hosts. Within this family, there are 8 distinct viruses known to cause significant disease in humans; among these are human herpesviruses 6A (HHV-6A), 7 (HHV-7), and 8 (HHV-8). HHV-6A and HHV-7 are members of the *Betaherpesvirinae* subfamily and the *Roseolovirus* genus. These viruses primarily infect young children, typically presenting with fever and rash; in immunocompromised individuals, the infection may progress to hepatitis, pneumonia, seizures, or encephalitis. HHV-8, also known as KSHV, is an oncogenic virus associated with Kaposi's sarcoma in immunocompromised patients. This virus has also been linked to primary effusion lymphoma, HHV-8-associated multicentric Castleman's disease, and KSHV inflammatory cytokine syndrome.

The accurate detection and quantification of these viruses during the early stages of infection are essential for managing clinical symptoms. However, viral growth and purification can be difficult, time-consuming, and costly. PCR-based methods provide an ideal alternative to traditional culture-based approaches; however, these molecular-based methods are dependent on the availability of high-quality reference materials. To address this need, ATCC has designed and developed quantitative synthetic molecular standards for HHV-6A, HHV-7, and HHV-8. As proof-of-concept, the HHV standards were quantified using Droplet Digital™ PCR (ddPCR™; Bio-Rad) and validated via published qPCR assays.[1,2,3]

ATCC Synthetic Molecular Standards

BSL-1	Quantitative	Stabilized

ATCC Part Number	Product Description
ATCC® VR-3264SD™	Quantitative Synthetic Human herpesvirus 6A DNA
ATCC® VR-3265SD™	Quantitative Synthetic Human herpesvirus 7 DNA
ATCC® VR-3261SD™	Quantitative Synthetic Human herpesvirus 8 DNA

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

References

1. Leibovitch EC, *et al*. Coinfection of human herpesviruses 6A (HHV-6A) and HHV-6B as demonstrated by novel digital droplet PCR assay. PLoS One 9(3): e92328, 2014. PubMed: 24663487
2. Dzieciatkowski T, *et al.* Human herpesvirus 7 in allogeneic hemopoietic stem cell transplant recipients in the Central Clinical Hospital in Warsaw: a three-year survey. Intervirology 54(1): 25-29, 2011. PubMed: 20689313
3. Lallemand F, *et al.* Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. J Clin Microbiol 38(4): 1404-1408, 2000. PubMed: 10747115

Materials and Methods

Quantitative Synthetic DNA
Using a proprietary method, we designed synthetic DNA constructs for HHV-6A (ATCC® VR-3264SD™), HHV-7 (ATCC® VR-3265SD™), and HHV-8 (ATCC® VR-3261SD™). These standards comprise fragments from various conserved regions of viral genomes, including the *U31* (large tegument protein), *U38* (DNA polymerase), *U57* (major capsid protein), *U65/U66*, *U67*, *U90* (IE1 / IE-A protein), and *U94* regions for HHV-6A; *U10*, *U31*, *U38*, *U39*, *U42*, and *U57* regions for HHV-7; and the minor capsid protein (*ORF 26*) and the latency-associated nuclear antigen (*LANA* or *ORF 73*) for HHV-8. Following their construction, the standards were authenticated via next-generation sequencing and quantified via droplet digital PCR.

qPCR Assay
qPCR assays were performed on the CFX96™ Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions with slight modifications.

Droplet digital PCR Assay
Droplet digital PCR assays were performed according to the manufacturer's instructions on the QX200™ droplet reader and QuantaSoft™ software 1.7.4.0917 (Bio-Rad) for droplet generation and data analysis.

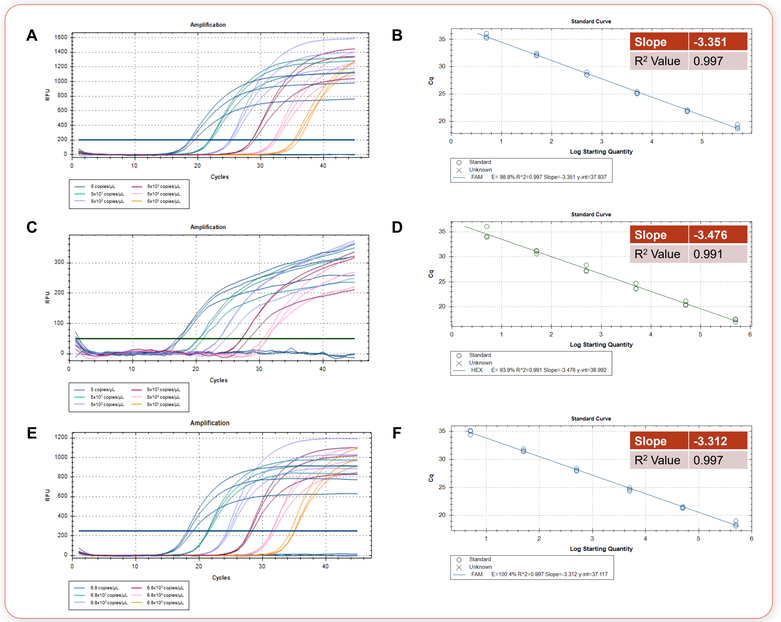

Figure 1. qPCR assay to verify the functionality of the synthetic molecular standards. (A, C, E) Amplification plots and (B, D, F) standard curves were generated with the HHV-6A, HHV-7, and HHV-8 molecular standards, respectively. The qPCR assays were performed as previously described.[1,2,3] Cycling conditions were 50°C for 2 min and 95°C for 2 min, followed by 45 cycles of 95°C for 15 sec and 59°C for 1 min for HHV-6A; and 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 1 min for HHV-7 and HHV-8. Standard curves were generated by using serial 10-fold dilutions that ranged from 5 to $5x10^5$ copies/µL for HHV-6A and HHV-7 and serial 10-fold dilutions that ranged from 6.8 to $6.8x10^5$ copies/µL for HHV-8. The DNA standards were tested in triplicate.

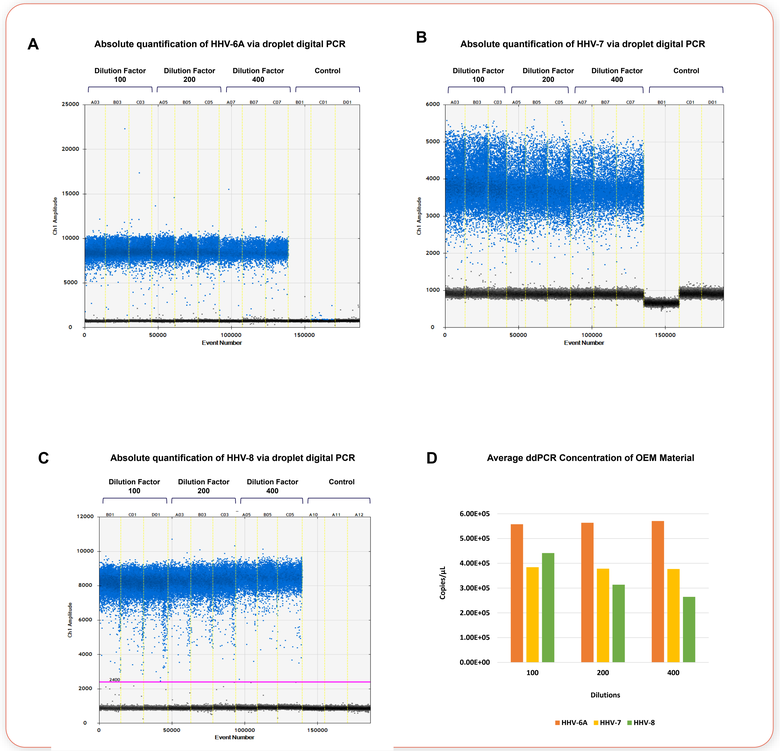

Figure 2. Absolute quantification of HHV-6A, HHV-7, and HHV-8 via droplet digital PCR. One-dimensional (1D) amplitude scatter plots of positive and negative droplet digital PCR reactions for 3 dilutions (dilution factors 100, 200, and 400) in triplicate for (A) HHV-6A, (B) HHV-7, and (C) HHV-8. HHV-6A, HHV-7, and HHV-8 were quantified by droplet digital PCR by using the same primers and probe from the appropriate published qPCR assays.[1,2,3] (D) Average calculated copy numbers/µL per dilution of stock (OEM) material. Droplet digital PCR was performed as follows: initial denaturation at 95°C for 10 min, amplified 40× at 94°C for 30 sec and 59°C for 1 min, and enzyme deactivation at 98°C for 10 min for HHV-6A; 95°C for 10 min, amplified 40× at 94°C for 30 sec and 60°C for 1 min, and enzyme deactivation at 98°C for 10 min for HHV-7 and HHV-8. Droplets were analyzed in the QX200 droplet reader. Data were analyzed with QuantaSoft software.

Conclusions

Our proof-of-concept data demonstrates that ATCC quantitative synthetic molecular standards for HHV-6A, HHV-7, and HHV-8 can be used as controls for assay development, verification, and validation. These standards were manufactured under ISO 13485 guidance and can be used to determine the viral load of unknown human herpesvirus samples through the generation of a standard curve. The standards are compatible with numerous published assays (7+, 4+, and 9+ published assays for HHV-6A, HHV-7, and HHV-8, respectively) 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.

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