External Validation of ATCC Molecular Standards for Herpes Simplex Virus (HSV-1 and HSV-2)

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Background & Introduction

- Herpes simplex virus (HSV-1 and HSV-2) causes a wide range of clinical manifestations that result in lifelong infections.
- Quantitative polymerase chain reaction (qPCR) assays are routinely used for the detection of HSV-1 and HSV-2 infections in clinical samples. However, the accuracy of a qPCR assay is dependent upon the generation of a standard curve using a positive control with a known genome copy number.
- Moreover, an independent positive control is required by clinical laboratories to monitor variations in assay performance for molecular assays.
- ATCC offers molecular standards for HSV-1 and HSV-2 for use as controls for the detection of these viruses from clinical samples and has recently developed a quantitative format for these standards for use in qPCR assays to detect and quantify HSV-1 and HSV-2 from unknown samples.
- To demonstrate the utility of quantitative molecular standards as controls in qPCR assays, we generated standard curves for each and quantified the working reagents for HSV-1 and HSV-2 from the National Institute for Biological Standards and Control, UK (NIBSC) as a proof-of-concept.
- Further, an independent validation study was performed in a clinical lab setting where the HSV-1 and HSV-2 molecular standards were run as controls alongside clinical samples in qPCR assays (n=36) to determine assay reproducibility.

Materials and Methods

Reagents:

The following reagents were used in this study:

- Molecular standards were prepared by extracting DNA from HSV-1 (McIntyre strain; ATCC® VR-539™) and HSV-2 (MS strain; ATCC® VR-540™) propagated in Vero cells (ATCC® CCL-81™), and the quantitative molecular standards (ATCC® VR-539DQ™ and ATCC® VR-540DQ™) were subsequently generated by determining the genome copy numbers of the respective molecular standards using Droplet Digital™ PCR (ddPCR™; Bio-Rad).
- The working reagent for HSV-1 and HSV- 2 were obtained from NIBSC, UK.
- Viral DNA from the respective working reagent for HSV-1 and HSV-2 was extracted using the QIAamp® Viral RNA Mini Kit (QIAGEN®).

qPCR Assay:

qPCR assays were performed using the quantitative molecular standards and the CFX96™ Real-Time PCR Detection System (Bio-Rad). The primer and probe sets from a published qPCR assay[1] were used, and the cycling conditions were 50ºC for 2 min, 95ºC for 2 min, followed by 45 cycles of 95ºC for 15 sec and 60ºC for 30 sec. Standard curves were generated using serial ten-fold dilutions of the respective quantitative molecular standard DNA, ranging from 2 copies to 2 x 10^5 copies/reaction for HSV-1 and for HSV-2. DNA 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).

Independent Validation of the ATCC Molecular Standards:

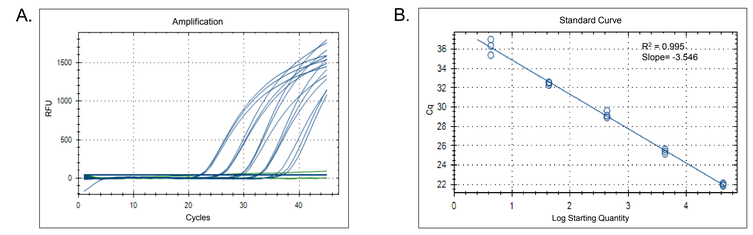
The ATCC molecular standards for HSV-1 and HSV-2 were serially diluted (1:10 - 1:10,000) and the optimal working dilution was determined to be 1:1000 using an in-house developed qPCR assay. To determine the reproducibility of the control material, as well as to establish a cycle threshold (CT) range, the controls were analyzed in a single replicate each time the HSV assay was run. Any qualitative test with Ct value of <40 was considered positive. The time frame for gathering this data spanned from February through April 2015, and 3 different technologists performed the HSV assay on different days. Statistical analyses and the X-bar graphs were created using GraphPad Prism software.

ATCC Molecular Standards	
ATCC® No.	**Description**
VR-539D™	Genomic DNA from Human herpesvirus 1 (HSV-1)
VR-540D™	Genomic DNA from Human herpesvirus 2 (HSV-2)
VR-539DQ™	Quantitative Genomic DNA from Human herpesvirus 1 (HSV-1)
VR-540DQ™	Quantitative Genomic DNA from Human herpesvirus 2 (HSV-2)

Results

ATCC Quantitative Molecular Standards

HSV-1 (ATCC® VR-539DQ™)



HSV-2 (ATCC® VR-540DQ™)

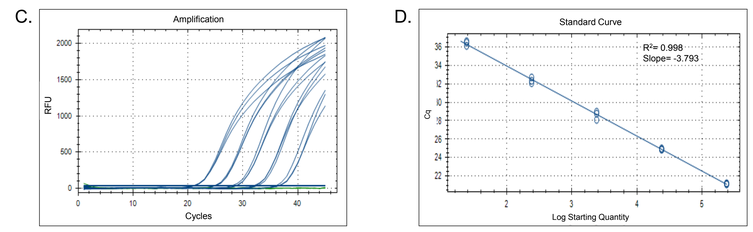


Figure 1. Generation of Standard Curves from ATCC Quantitative Molecular Standards for HSV-1 and HSV-2. A&C) Amplification plots and **B&D)** standard curves generated using the HSV-1 and HSV-2 quantitative molecular standards (ATCC® VR-539DQ™ and ATCC® VR-540DQ™, respectively) with the primer and probe set from the published qPCR Assay[1]. (Blue) = Serial ten-fold dilutions of the quantitative molecular standard. (Green) = Negative control.

Utility of ATCC Quantitated Molecular Standards as Controls

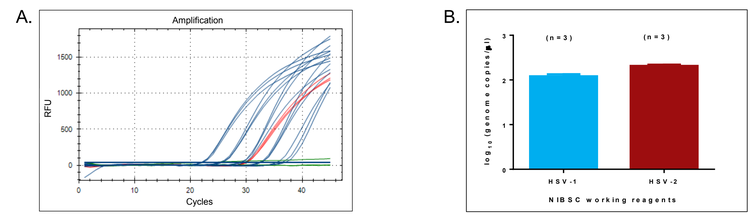
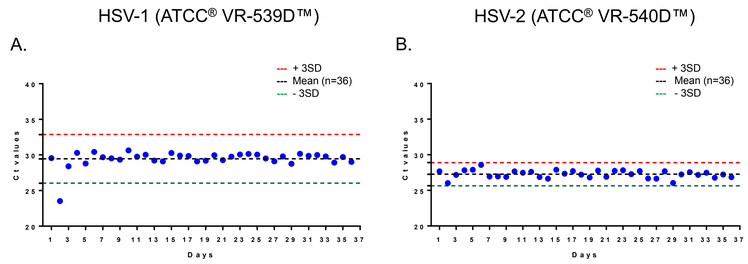


Figure 2. Quantification of NIBSC Working Reagents for HSV-1 and HSV-2 Using ATCC Quantitative Molecular Standards. A) An example of a qPCR amplification plot showing the ATCC quantitative molecular standards (Blue), the NIBSC working reagent for HSV-1 (Red), and a negative control (Green). **B)** Quantitation of the working reagent for HSV-1 (Blue) and HSV-2 (Red). Both working reagents were run in triplicate wells (n=3) using the HSV-specific published assay[1]. The average from triplicate wells were used to calculate the quantities of the HSV-1 and HSV-2 genomes, respectively. Error bars indicate standard deviation and were calculated using GraphPad Prism software.

Independent Assay Validation of Molecular Standards



C.

ATCC Molecular Standards	Average CT	Standard Deviation (SD)	Co-efficient of Variation (CV)
HSV-1 (ATCC® VR-539D™)	29.46	1.14	3.9%
HSV-2 (ATCC® VR-540D™)	27.27	0.54	2.0%

Figure 3. Independent Validation of the Molecular Standards for HSV-1 and HSV-2. HSV-1 and HSV-2 molecular standards (ATCC® VR-539D™ and ATCC® VR-540D™, respectively) were diluted 1:1000 and run as positive controls with the lab-developed qPCR assays over a period of 3 months with 3 different operators (n=36) at the Cincinnati Children's Hospital Medical Center. CT values were obtained during each run from **A)** HSV-1 and **B)** HSV-2 molecular standards, and were plotted against the number of days to show the run-to-run reproducibility of the positive controls using GraphPad Prism software. **C)** The values for the average CT, standard deviation, and % CV, 3.0 SD range were calculated using GraphPad Prism software. The clinical laboratory considered all Ct values < 40 as positive for HSV.

Conclusions

- The quantitative molecular standards provide well-characterized reference materials for qPCR assays for the detection and precise quantification of HSV-1 and HSV-2.
- The quantitative molecular standards can be readily used to assign a genome copy number to secondary calibrators or unknown samples.
- These standards are compatible with published assays for HSV-1 and HSV-2 and can be used as controls for assay development, verification, and validation.
- External validation of molecular standards for HSV-1 and HSV-2 by a clinical laboratory indicates that these standards can be successfully used for monitoring day-to-day variation and assay performance.

Reference:

1. Ryncarz AJ, *et al*. Development of a high-throughput quantitative assay for detecting herpes simplex virus DNA in clinical samples. J Clin Microbiol 37(6): 1941-1947, 1999. PubMed: 10325351

Disclaimers